FORMIMINOGLUTAMIC ACID URINE EXCRETION

Clinical studies in individuals exposed to nitrous oxide

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For degree of Doctor of Medicine
University of Edinburgh
1993

For Sarah

CONTENTS

DECLARATION

This thesis has been written by myself. The first four and the last chapters are based on personal surveys of the literature whilst the rest of the thesis describes clinical studies performed mainly by myself with assistance from colleagues in both the Departments of Anaesthesia and Clinical Chemistry, Edinburgh and the Department of Anaesthesia, Glasgow. In all cases, I was the principal investigator.

ABSTRACT

A brief history of the discovery of the anaesthetic potential of nitrous oxide is given followed by a more detailed history of the discovery of its specific toxic depressant effect on bone marrow. The work demonstrating that this effect is due to nitrous oxide reacting with vitamin B_{12} and, consequently, inhibiting methionine synthase is described. Because the clinical results of this enzymatic inhibition in inhibiting DNA synthesis are mediated through both the folate and methionine metabolic pathways, these are discussed. Various theories on how this mediation occurs are contrasted.

The clinical relevance of these potential toxic side effects are discussed. Methods of assessing the toxicity of nitrous oxide are analysed and it is concluded that they are either too insensitive or require highly invasive sampling. The use of the formiminoglutamic acid urine excretion test in assessing folate metabolism is appraised and the possibility of using this test to assess nitrous oxide toxicity evaluated. Raised excretion is an index of folate metabolic abnormalities.

This thesis then examines the toxicity of nitrous oxide in clinical practice by using this test. Firstly, a reproducible and accurate assay system for urinary formiminoglutamic acid was designed and values for its excretion in normal individuals determined. Then the

amount excreted in subjects exposed to nitrous oxide under a variety of conditions was found. It was demonstrated that exposure to nitrous oxide during anaesthesia has deleterious consequences on folate metabolism at an exposure dose lower than was previously though because subjects were found to excrete larger amounts of formiminoglutamic acid for the first 2 days after exposure to nitrous oxide. Excretion was raised with exposures of greater than 90 minutes. Patients with cancer, who theoretically are more susceptible to the toxic affects, are similarly affected, but not to a significantly greater degree. Chronic exposure of anaesthetists due to nitrous oxide theatre pollution appears to have no effect. Nitrous oxide therefore has a specific toxic effect at lower doses than has been reported.

Finally, the place of nitrous oxide in modern anaesthesia is discussed. Its advantages and disadvantages are evaluated and the problems that may occur if it was withdrawn from use in anaesthesia. The conclusion of this thesis is that nitrous oxide due both to its specific toxic effect on vitamin B_{12} and to its overall incidence of other side effects has a poor cost-benefit index and that it may be of advantage to the patient if it was gradually withdrawn from use.

CHAPTER ONE

NITROUS OXIDE AND METHIONINE SYNTHASE

Introduction

Nitrous oxide (N_2O) is used in most modern general anaesthetics because it is believed to have many advantages and only a few minor problems. However, evidence that has accumulated since 1978 contradicts this view because N_2O is now known to have a specific inhibitory effect on DNA synthesis. This chapter will give a brief résumé of the history of N_2O usage in anaesthesia, describe how its bone marrow depressant effect was discovered and indicate how it may occur. The second chapter will describe folate and methionine metabolism because they are central to N_2O toxicity. The different theories on how abnormalities in folate metabolism can cause marrow depression will be examined.

Brief history of the use of Nitrous oxide in anaesthesia

Nitrous oxide was probably first discovered by Joseph Priestley in 1772, after work by Dr Stephen Hale. Priestley had added Walton pyrites (iron disulphide) to nitric acid to form a red mixture that absorbed air forming "nitrous air". Priestley found that the volume of this air decreased when it was added to a water paste of iron filings and brimstone. The gas formed preserved animal substances from putrefaction and was lethal if given to animals.

Thomas Beddoes and Humphrey Davy established the Pneumatic Medical Institute in Bristol in 1799 to practise

Pneumatic medicine. Davy noted that N_2O inhalation reduced the pain of toothache and gave pleasurable, excitable sensations. He suggested that it had potential as an anaesthetic although he took this no further. In America, Gardner Colton gave a demonstration of this new gas as a circus sensation. A local dentist, Horace Wells, noticed that a drugstore clerk, Samuel Cooley, while under its influence banged his leg, but felt no pain. He reasoned that it might prevent pain occurring during dental extraction and to test this, he inhaled N_2O during the removal of one of his own teeth. He found that it was an effective analgesia and experimented on a further 15 patients with partial success. However, Wells attempted to exhibit its analgesic properties in front of a medical audience in Boston, but the demonstration was a failure and Wells was booed out of theatre.

Ether and chloroform were being tested as potential anaesthetics and were found to be more reliable than N_2O and so were used in preference. However, in 1863, Gardner Colton revived the usage of N_2O in dentistry and was successful in thousands of cases. Since then, it has been used in many patients. It has been extensively investigated and found to have only a few disadvantages. It is a weak anaesthetic, it diffuses into closed spaces causing either an increase in size or pressure of the closed space and it causes hypotension in shocked patients. These effects were felt to be minor and obvious. However, a new, less predictable effect has been discovered which may be much more serious.

Nitrous oxide causes bone marrow depression

The first sign that N_2O had a specific effect was seen in the mid 1950's when Lassen and others (1954) published 4 case histories describing a treatment for tetanus which used curare to muscle relax the patient. This was a new technique and because it was possible that the patient could be conscious whilst paralysed, various sedatives were used. Inhalation of 50% N_2O in oxygen was found to be successful. However, Lassen reported the death of one patient and severe bone marrow depression occurred in another two although this was initially thought to be due to a side effect of the tetanus toxin.

Gormsen (1956) described the haematological depression that occurred during the treatment of a patient who had died 22 days after admission for tetanus. He had been curarised and ventilated with N_2O. The other drugs he received included penicillin, terramycin, achromycin, chlorpromazine and cortisone. Granulocytopenia, leucocytopenia and thrombocytopenia all developed and he needed multiple blood transfusions to prevent anaemia. It was thought that curare was responsible.

Lassen and others (1956) examined another 13 cases of bone marrow depression occurring during this treatment. Six patients had developed abnormal megaloblastic marrows and their neutrophil and platelet counts were low. These findings are similar to those of untreated pernicious anaemia. The other 7 remained normal. Because the severity

of the disease was similar in both groups, it was considered unlikely that the depression was due to tetanus itself. The only drug common to all 6 patients was N_2O. Curare was used in only 3 of them.

It therefore appeared that N_2O was responsible. To conclusively prove this, 2 patients were given it in a trial. In both cases their haematological profile became megaloblastic after inhaling N_2O for two or three days and this became normal a few days after removing the gas. It appeared that N_2O could in some unknown manner cause bone marrow depression if given for a long time.

Other work showed a similar effect (Wilson and others, 1956, Sando and Lawrence, 1958). Stead and others (1962) described a 10 year old boy who had received 50% N_2O intermittently over 23 days with a total exposure of 90 hours. Leukopenia developed after 21 days. On day 25, N_2O was stopped and his marrow was normal by day 33.

Interestingly, animal experiments failed to show similar results (Lassen and Kristensen 1959). N_2O was found to be cytotoxic to embryonic cells. Because of this, Lassen and Kristensen (1959) gave it to 2 patients suffering from chronic myeloid leukaemia in an attempt to kill the cancer cells. They found that prolonged inhalation did decrease cell numbers, but that they rapidly returned to normal when N_2O was stopped. In addition, both had changes in their mental state experiencing elation, depression, lassitude and eventually coma. These mental changes were the first description of the effects that N_2O can have on the nervous system.

How N_2O could cause this haematological depression was unknown although it was initially considered that impurities were responsible. Higher oxides of nitrogen including nitric oxide and nitrogen dioxide were present in the gas and workers exposed to these contaminants were known to have developed leukopenia. It was thought that the depression represented mild chronic poisoning (Parbrook 1976). In addition, the leukopenia might have been caused by either increased cell destruction or altered distribution of the peripheral cells. Johnston and others (1971) showed that erthropoietic depression occurred within 2 days when rats were exposed to 80% N_2O. They measured the distribution of radioactive ^{59}Fe and ^{14}C-thymidine (indices of erythropoeisis and nucleic acid synthesis respectively) in the blood cells, liver, spleen and marrow. Depression occurred initially in the spleen and bone marrow and only afterwards in the peripheral blood suggesting that N_2O mainly affects the stem cells and causes a decrease in cell synthesis. A decreased response to exogenous erythropoietin in these rats further supported this view.

Kreike and others (1977) exposed rats to either 0, 20 or 40% N_2O for 35 days. With the highest dose, bone marrows were depressed within 3 days whilst it took 14-21 days with 20% N_2O. Recovery occurred about 3 days after N_2O was withdrawn. In intermittently exposed rats a tolerance developed to 40% N_2O after 10 days. Therefore it appeared that this bone marrow depression was time and

concentration related and that rats have a mechanism of bypassing it that takes a few days to develop.

Nitrous oxide has been given safely over long periods

During 1974-1976, Amess and his colleagues noticed that 27 patients had developed acute megaloblastic bone marrow changes 2-14 days after admission to an Intensive Care Ward. Seventeen had been ventilated with 50% N_2O for 1-8 days and the deoxyuridine suppression tests (dU test) of some of these patients suggested a diagnosis of untreated vitamin B_{12} deficiency. However, all had normal plasma vitamin B_{12} concentrations (Amess and others 1976).

There were many possible reasons for this marrow depression (Editorial 1982) although, it seemed probable that N_2O was, at least, partially responsible. However, N_2O had been inhaled previously over long periods with no problems. Petrovsky and Yefuni (1965) had given 30-70% N_2O for post-operative pain relief to 102 patients for up to 96 hours. Their peripheral blood remained normal although bone marrow was not examined. Thompson (1976) gave 35% N_2O to 42 patients for up to 48 hours to relieve the pain of myocardial infarction. No bone marrow depression occurred. A patient was treated for pentazocine addiction by inhalation of N_2O. He received it continuously for 30 days, and then for 18 hours daily for the next 15 days and finally for 6 hours daily for a further 202 days. No abnormalities were seen in the first 92 days although he was given a multivitamin preparation.

After discharge, he developed folic acid deficiency which responded to oral replacement. Interestingly, 2 months later he developed a neurological deficit which was diagnosed as a vitamin deficiency and which responded to vitamin B complex therapy (Kripke and Hechtman 1972). The finding that some individuals can inhale N_2O for a long time without developing signs of marrow depression complicates the issue of N_2O toxicity.

Nitrous oxide and the deoxyuridine suppression test

To further investigate the marrow depressant effects of N_2O, Amess and his colleagues (1978) designed a trial involving the postoperative ventilation of 22 cardiac surgical patients for 24 hours. Each subject was allocated to one of three groups.

1) 8 received 50% N_2O in oxygen for the whole period.

2) 9 received 50% N_2O in oxygen for the operation (5 - 12 hours) followed by an air/oxygen mixture for 18 hours.

3) 5 received no N_2O, but an air/oxygen mixture for 24 hours with additional phenoperidine during the operation to compensate for the lack of N_2O.

Bone marrow was aspirated before induction, 24 hours later and in some cases after 6 days. Marrow was assessed in 2 ways, subjectively by using microscopic examination

and objectively by the dU test. A peripheral blood film was examined daily.

The dU test assesses the ability of marrow cells to manufacture fresh DNA and is used in the diagnosis of abnormal vitamin B_{12} and/or folate metabolism (Metz and others 1968, Das and others 1970, Van der Weyden 1973, Wickramasinghe and others 1986). Deoxythymidine is one of the 4 essential bases in DNA and marrow cells can make it by one of two pathways;

a) Using thymidine synthase (TS) and a tetrahydrofolate (THF) co-enzyme. This is the main method of deoxythymidine synthesis.

Deoxyuridine + 5,10 MethyleneTHF ----> Deoxythymidine + Dihydrofolate

b) The salvage pathway in which thymidine kinase converts thymidine to deoxythymidine.

Thymidine + ATP ----> Deoxythymidine + AMP + Pi

The marrow uses the TS pathway preferentially, but is able to use the salvage pathway when there is a shortage of 5,10 methylene THF (as in vitamin B_{12}/folate deficiency).

Incubating deoxyuridine with marrow cells results in deoxythymidine production by TS and if radioactive ^{3}H-thymidine is then added, little will be converted to deoxythymidine by the salvage pathway and so only small amounts of the new DNA will be radioactive. If there is insufficient 5,10 methyleneTHF present to make enough DNA

for the cell, it produces the rest from the salvage pathway. ^{3}H-thymidine will be incorporated into new DNA and its radioactivity will be increased. The greater the deficiency of 5,10 methyleneTHF, the greater the incorporation of radioactivity into the cells. The percentage,

$$\frac{\text{H3-thymidine in DNA with deoxyuridine added} \times 100\%}{\text{H3-thymidine in DNA with NO deoxyuridine added}}$$

is the dU test figure and for normal marrow it is less than 10% although each laboratory has its own limits. Figures greater than 10% indicate a shortage of 5,10 methyleneTHF due to abnormalities in either folate or vitamin B_{12} metabolism. Differentiation between the two can be made by adding a small amount of methylcobalamin or folic acid to the cells. With vitamin B_{12} deficiency, methylcobalamin partially corrects the dU test whilst folic acid has no effect and conversely for folate deficiency. A correction of greater than 15% indicates a vitamin deficiency.

In the study of Amess and others (1978), patients in group 3 had normal marrows throughout. However those in group 1 all had megaloblastic marrows after 24 hours which were identical to those seen to untreated pernicious anaemia. The dU tests were abnormal and were corrected by methylcobalamin. After 6 days all the dU tests performed were normal indicating that the depression was reversible. In group 2, 3 patients had mildly megaloblastic marrows

after 24 hours and 3 had abnormal dU tests, again all characteristic of vitamin B_{12} deficiency. However because all patients had normal vitamin B_{12} concentrations, it suggested that the problem is in vitamin B_{12} metabolism. Somehow exposure to N_2O had caused vitamin B_{12} to function abnormally.

Nitrous oxide oxidises cobalt

The work of Banks, Henderson and Pratt (1967) suggested an explanation for this abnormality. They were chemists investigating the effects of N_2O on transition metals and had found that in vitro, N_2O could oxidise cobalt from its monovalent to its bi and trivalent states.

$$2N_2O + 2Co(I) + 2H2O \text{ -----> } 2Co(III) + 2N2 + 4OH^-$$

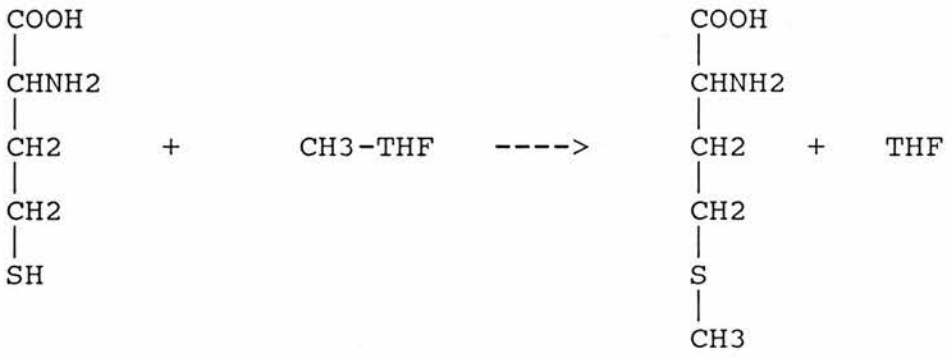
$$Co(I) + Co(III) \text{ -----> } 2Co(II)$$

Their work had been published in a chemical journal not commonly read by anaesthetists and its significance was not understood until the work of Amess and his colleagues was published.

Cobalt is the essential co-factor of vitamin B_{12}. Vitamin B_{12} is a complex molecule (Davis 1985) composed of a central corrin ring with cobalt at its centre. At right angles to this ring is a nucleotide 5,6 dimethyl-benzimidazole. One nitrogen atom from this molecule is attached to the corrin ring with the other being bonded to the cobalt. In vitro, various compounds can bond at the

6th position of the cobalt atom (cyanide, hydroxyl, deoxyadenosyl or methyl groups). In humans, this vitamin is involved in only 2 reactions.

1. Transfer of a methyl group from 5-methylTHF to homocysteine to produce methionine, catalysed by methionine synthase (MS). The vitamin is in its methyl-cobalt form and the enzyme is not found in the blood.



Homocysteine + 5 methylTHF ----> Methionine + THF

2. Conversion of methylmalonyl CoA to succinyl CoA by an isomerase reaction. Methyl-malonyl CoA mutase (MMCoA mutase) catalyses this reaction and vitamin B_{12} is in its adenosyl-cobalt form.

Methyl-malonyl CoA ----> Succinyl CoA

Therefore, if the marrow depressant effect of N_2O is due to an abnormality of vitamin B_{12} metabolism, then it could be secondary to inhibition of either, or both enzymes.

Nitrous oxide inhibits methionine synthase

Deacon and others (1978) exposed Sprague-Dawley rats to 50% N_2O for up to 24 hours and assayed the MS activity directly by incubating rat livers with homocysteine and 5[^{14}C]methylTHF acid. Measurement of the radioactive (^{14}C) methionine formed gives an indication of activity. MMCoA mutase was assayed indirectly by measuring the formation of methylmalonic acid in the urine because if this enzyme is inhibited, the concentration of this compound increases. Thirty minutes N_2O exposure substantially reduced MS activity and after 6 hours it was totally inactive. In contrast, methylmalonic acid excretion was not increased after 24 hours exposure even if its catabolic pathway was stressed by an intraperitoneal injection of proprionic acid, a precursor of methylmalonic acid. Hence it appeared that N_2O inhibited MS and not MMCoA mutase.

Other work has shown similar results. Kondo and others (1981) exposed Sprague-Dawley rats to 80% N_2O for up to 792 hours. MS activity was assayed as above and MMCoA mutase activity was found by measuring the amount of [^{14}C]succinyl CoA formed from 2[^{14}CH3]methylmalonyl CoA. Fifteen minutes and 1 hour N_2O exposure caused MS activity to decrease by 55% and 70% respectively. No further decreases occurred after this. No change in MMCoA mutase activity was seen in the first 48 hours although it did decrease after 384 and 792 hours (to 48 and 32% of controls respectively). With these long exposure times,

there was also a decrease in tissue cobalamin concentrations suggesting a deficiency of vitamin B_{12} which results in inadequate manufacture of MMCoA mutase.

Methylmalonic acid excretion was measured in 5 humans ventilated with 50% N_2O for 24 hours (Rask and others 1983). Urine was collected before, during and after ventilation. Methylmalonic acid excretion increased by 300% during N_2O exposure. However, no suitable control group was used and the increase could have been due to an anaesthetic or surgical cause. In addition, a relatively non-specific calorimetric assay was used to measure methylmalonic acid. It is probable that MMCoA mutase inhibition is too slow to explain the clinical effects of N_2O.

Is MS inhibited by a specific effect of N_2O or due to a general anaesthetic effect?. Deacon and others (1980) exposed rats to 100% oxygen and 0.35% halothane for 6 hours and showed only a slight decrease in MS activity. Koblin et al (1981) showed that there was no decrease in MS activity when mice were exposed for 4 hours to halothane (0.69%), enflurane (1.02%) or isoflurane (0.6%). It appears that anaesthesia itself is unable to inhibit MS and N_2O alone has this specific effect.

Species difference in MS Inhibition

Is MS of other species inhibited by N_2O? Koblin and others (1981) showed that the activity of liver MS in CD-1

mice who had been exposed to 80% N_2O for 4 hours decreased by 95%. Ethically it is more difficult to assess humans. Kano and others (1981) studied 22 patients (7 anaesthetised without N_2O and 15 with) by aspirating bone marrow before and after anaesthesia and found that MS activity in the N_2O exposed group became abnormally low after 4 hours with an inactivation $t_{\frac{1}{2}}$ of 1.5 to 6 hours. Koblin and others (1982) were able to take timed liver samples from 14 patients undergoing laparotomy. Seven were given 50-70% N_2O and the rest acted as controls. Liver biopsies taken at different times demonstrated a dose dependent (time of N_2O x concentration) decrease in MS activity.

Landon and Toothill (1986) measured MS activity in human placental tissues from 11 patients undergoing Caesarean delivery (N_2O exposure varying from 13-22 minutes) and from 20 patients undergoing vaginal delivery (no N_2O given) and found no difference. Perhaps human MS is resistant to N_2O and therefore longer exposures are needed before inhibition occurs.

In an attempt to quantify the rate of human liver MS inactivation, Royston and others (1988) anaesthetised 22 patients with 70% N_2O. Liver biopsies were taken at variable times during the operation and the MS activity they possessed was assayed. Batches of 6 Sprague-Dawley rats exposed to 50% N_2O in oxygen for 5, 10, 20, 40 and 80 minutes also had liver MS activity assayed. Human MS activity varied greatly, but it tended to decline with an increasing duration of exposure. A mean inactivation $t_{\frac{1}{2}}$ of

46 minutes was found compared to 5.4 minutes for rats. Hence, it appears that N_2O does inactivate MS from different species although at different rates and humans appear to be less susceptible to its effects. However, as will be discussed, human individual MS activity does vary greatly and it is possible that the inactivation $t_{\frac{1}{2}}$ may be wrong.

Inhibition of MS from different tissues

MS is found in many tissues except blood and it is always inhibited by N_2O. MS activity in the brain of Sprague-Dawley rats exposed to 50% N_2O for 1 to 15 days was almost undetectable (Deacon and others 1980) and a similar decline was seen in the brains of CD-1 mice (Koblin and others 1981) and the kidneys of rats (Kondo and others 1981). Kano and others (1981) showed that bone marrow MS was inhibited by N_2O. MS activity in rat testis was inhibited by exposure to 10% N_2O for 1 hour (Brodsky and others 1984). Thus it appears that MS from all tissues is similarly affected.

MS activity

Individual MS activity appears to vary greatly both in animals and humans. Activity is defined as the amount of methionine formed per unit time per mass of protein. Sprague-Dawley rats, exposed only to air had large variations in their liver MS activity. Different values

include 2.0 (S.E.M ± 0.6) nmol methionine.hr^{-1}.mg $protein^{-1}$ (Deacon and others 1980), 1.4 - 3.4 nmol methionine.hr^{-1}.mg $protein^{-1}$ (Deacon and others 1978) and 1.5 -3.2 nmol methionine.hr^{-1}.mg $protein^{-1}$ (Nunn and others 1982). A similar wide range was seen in mice, ranging from 300 to 600 nmol methionine.hr^{-1}.g $liver^{-1}$. Humans variability is equally great. Koblin and others (1982) showed it to range from 285 to 523 nmol methionine.hr^{-1}.g $liver^{-1}$. Royston and others (1988) found human MS activity to range from 0.85 to 1.95 (mean 1.16, SEM 0.2) nmol methionine.hr^{-1}.mg $protein^{-1}$. In addition, they found great individual variation in the rate of MS inactivation by N_2O. Landon and Toothill (1984) showed the activity in 20 placentae to range from 0.8 to 2.6 (mean 1.7) nmol methionine.hr^{-1}.mg $protein^{-1}$.

Hence the rate that MS is inhibited by N_2O is not constant. The wide range of MS activity that occurs in normal patients indicates that large numbers of patients need to be studied for statistically relevant results.

Inhibition of MS by trace concentrations of N_2O

Is MS inactivated by low concentrations of N_2O? Kondo and others (1981) showed that Sprague-Dawley rats exposed to 2% N_2O for 15 hours had a 30% decrease in liver MS activity. Koblin and others (1981) studied the chronic effects on CD-1 mice of different concentrations of N_2O. Exposure to less than 5% N_2O for 4 hours had no effect

whilst 10% N_2O caused a 50% reduction in its activity. Additionally rats exposed to 700 - 1,600 ppm N_2O (average 1,100 ppm) for up to 22 days had their MS activity reduced by 73% after 8 days although it became normal with longer exposures.

Sharer and others (1983) measured the dose-effect relationship between chronic exposure of Sprague-Dawley rats to N_2O and liver MS activity. The rats were exposed in groups of 4 to 15 animals to 500, 1000, 2500, 5000, 10,000, 20,000 and 50,000 ppm N_2O for 1 to 28 days. The lowest N_2O concentration that caused any MS inactivation after 24 hours was 1000 ppm and for longer times it was 500 ppm. Extrapolation of these results indicate that the minimum N_2O concentration which causes any MS inhibition over 24 hours is 840 ppm and for longer, 450 ppm. The ED50 for N_2O induced MS inactivation for 24 hours was 10,400 ppm and for 2 - 28 days was 5,400 ppm.

Inhibition of MS by intermittent exposure to N_2O

The effect that intermittent N_2O exposure has on animals has been assessed in Sprague-Dawley rats. Nunn and others (1982) gave 2 groups 50% N_2O in 50% oxygen for three 15 minute periods each day for 4 and 21 consecutive days. Liver MS activity in the 4 day group was 39.4% of controls and in the others was 20.4%. N_2O therefore has a cumulative effect if used intermittently although the duration of exposure needed for inhibition is still unknown.

Recovery of MS inhibition

MS recovery after inhibition is slow. Deacon and others (1980) showed that rats exposed to 50% N_2O for 6 hours had a MS activity of only 50% normal after 60 hours and after 84 hours, 1 of 2 rats still had abnormal activity. Kondo and others (1981) confirmed this slow recovery by exposing rats to the same concentration of N_2O for 15 hours. After 6 and 72 hours, MS activity was 29% and 80% respectively when compared to controls. In addition, they showed that its recovery paralleled the reappearance of cobalamin bound to MS. Slow recovery taking 2-4 days was seen in mice exposed to 0.8 atm N_2O for 4 hours. After 24 hours, only 70% recovery occurred (Koblin and others 1981).

Possible mechanisms of MS inhibition

Why recovery of MS activity should take so long is still unknown. It is possible that MS inactivation is irreversible and new enzyme must be synthesised. Addition of exogenous vitamin B_{12} has little effect on MS activity indicating that the B_{12} ligand is tightly bound to MS (Coward and others 1975). The irreversibility of this inactivation may be due to how it occurs. Frasca and others (1986) examined purified E. coli and partially purified pig liver MS. N_2O inactivation *in vitro* only

occurred when the enzyme was in its active form. Pre-incubation of MS with N_2O when its cobalt was in a Co(II) inactive form did not result in inhibition because it could easily be converted to Co(I) by a reducing system and then was fully active. However, when cobalt was in its Co(I) form, N_2O caused irreversible inactivation. They postulated that inactivation was not caused by oxidation of Co(I) to Co(II) and Co(III) because this could not explain the irreversible nature of the process. A reduction system should have been able to convert Co(II) back to Co(I). Possibly, Co(III) could react with an amino group of an amino-acid to form a stable complex although this interaction would give a Co(III) absorption spectra when a Co(II) was seen. Frasca postulated that during N_2O inactivation, a free hydroxyl radical ($OH^{\cdot}$) was produced;

$$Co(I) + N_2O + H^+ \text{----->} Co(II) + N2 + OH^{\cdot}$$

Free radicals are atoms or molecules, existing independently and contain one or more unpaired electrons which mahes them highly reactive. A hydroxyl radical has 9 protons and electrons with one unpaired electron in its outer shell. In cells, proteins, nucleic acids, membrane lipids and supporting matrices are attacked. Proteins containing high concentrations of sulphur containing amino-acids are especially vulnerable to deprotonisation by $OH^{\cdot}$ (Royston 1988). Nearly 4% of the amino acids in MS contain sulphur and many of these are near the active site of the enzyme and when $OH^{\cdot}$ is formed, it can react with

them to produce irreversible inactivation of the enzyme (Fig. 1).

There is further evidence that OH· is produced by the interaction of N_2O and cobalt. Koblin and others (1990) examined MS activity in the livers, kidneys and brains of mice exposed to N_2O and studied the effects that dimethylthiourea (DMTU) had. This is a potent hydroxyl radical scavenger which readily penetrates cell membranes. The mice were given either intraperitoneal injections of DMTU or saline and then exposed to N_2O for 30 minutes, 2, 4 and 8 hours. Enzyme inactivation was significantly less in the DMTU group and this this was more noticable with longer N_2O exposure times. A second experiment showed that there was a DMTU dose dependent effect on inactivation. DMTU gave protection for up to 4 hours after injection and after N_2O exposure, injected DMTU slowed the rate of recovery (Koblin and Tomerson 1990).

This series of experiments supports the concept of MS inactivation after OH· formation. DMTU may protect MS by reacting with any OH· formed by N_2O and thereby prevent it from reacting with amino-acids. It does not inactivate all OH· because inactivation will eventually occur and once this happens, DMTU has no benefial effect. This proposed mechanism of MS inactivation can explain the species difference in MS inactivation $t_{\frac{1}{2}}$. An intrinsic cytoplasmic hydroxyl scavenging system may exist offering varying degrees of protection between different species.

It is possible that the marrow depression caused by N_2O may be due to the metabolism of N_2O in the body to

toxic compounds although only a small amount is actually metabolised. Sawyer and others (1972) found that the liver removed only 0.03 ± 0.05% of N_2O passing through it. In vitro exposure of human faeces to N_2O (with 5% oxygen) for 16 hours resulted in 47 nmol of N2 (SD 13) being produced per gram wet sample. Calculations show that if a 70 kg man breathed 75% N_2O for 3 hours, only 1.0-1.3 ml N2 would be produced from N_2O. The presence of oxygen inhibits any metabolism of N_2O (Hong and others 1980) and it is possible that free radicals are formed during this metabolism (Bosterling and others 1980). Hence some of the clinical effects of N_2O may be due to this metabolism, but they are more likely to be caused by MS inhibition.

Summary of MS inhibition

The initial use of N_2O in anaesthesia and how it was discovered that it caused marrow depression has been described. This effect is a result of N_2O inhibiting MS in a time and concentration dependent manner. It occurs in all tissues and in all species tested, is irreversible, cumulative and may only require trace concentrations of N_2O. Recovery of activity is slow. Inhibition of the enzyme may be by hydroxyl radical formation.

How does inhibition of this enzyme cause bone marrow depression and its other possible effects? To understand this, folate metabolism needs to be discussed because it is intimately involved in vitamin B_{12} metabolism.

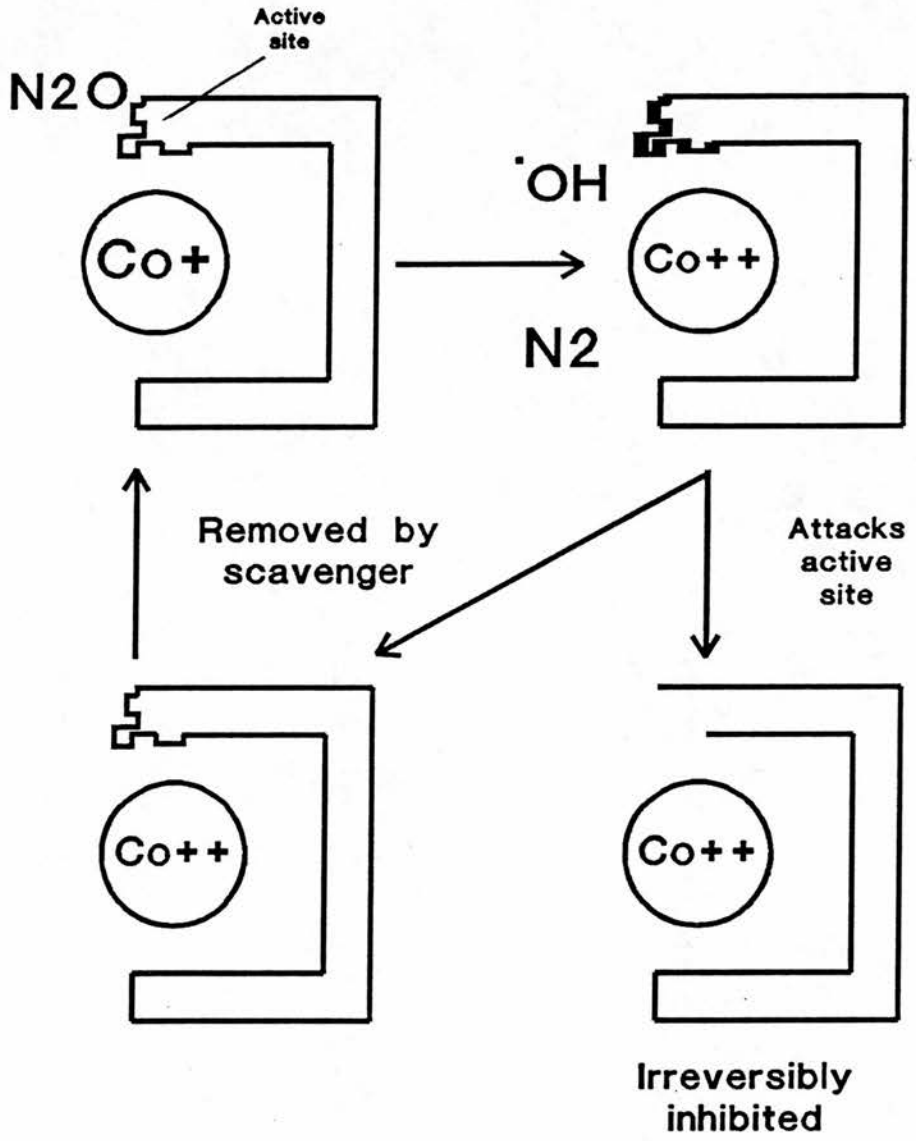


Figure 1. Inactivation of methionine synthase by N_2O due to formation of a hydroxyl radical.

CHAPTER TWO

FOLATE AND METHIONINE METABOLISM:

Mechanisms of toxicity

Introduction

It was first realised that folic acid was an essential vitamin in 1930 when an autolysed yeast preparation "marmite" cured pregnant Indian women who suffered from a macrocytic anaemia (Wills 1931). Subsequent work identified the active factor as pteroylglutamic acid and this was first synthesised in 1945 (Angiers 1945). Folic acid deficiency is common and its clinical symptoms are similar to those of vitamin B_{12} deficiency. There is a generalised disorder of cellular metabolism and a macrocytic megaloblastic anaemia occurs. These two vitamins and methionine, an essential amino-acid are biochemically closely interrelated as will be discussed in this chapter.

Structure and activity

Folic acid can be metabolised different co-enzymes, all which function by accepting or donating one-carbon fragments to various compounds (Shane and Stokstad 1985). Structurally, there are 3 distinct parts (Fig.2).

1) Pteridine moiety which binds the one carbon fragments

2) para amino benzoic acid - joined to 1) by a methylene bridge

3) Glutamate residues - can have any number from 1 to 10. They are linked by an alpha amino

group to the gamma carboxyl group. The monoglutamate is the transport form with the polyglutamate being the active form.

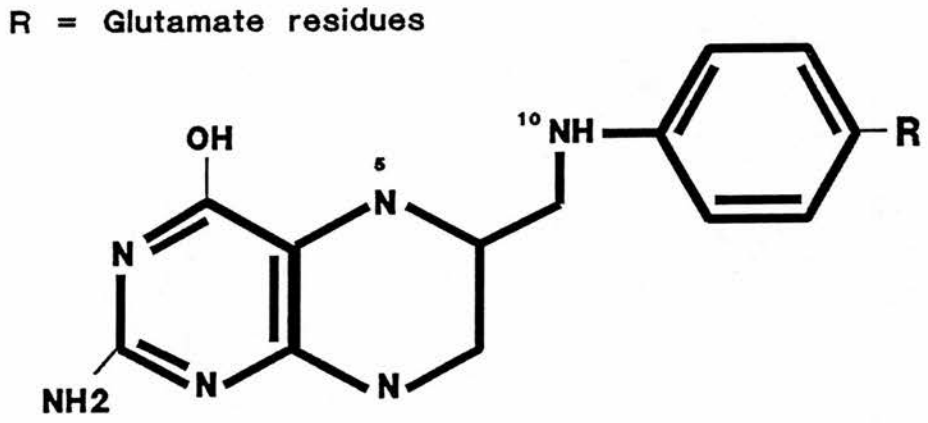


Figure 2. Structure of tetrahydrofolic acid.

The one-carbon fragments that bind to folic acid are at different oxidative levels;

Formate -CHO bound at the N5 or N10 atom

2. Methenyl =CH- bound to the N5 and N10 atoms

3. Methylene -CH2- bound at the N5 and N10 atoms

4. Methyl -CH3 bound at the N5 atom

Folate can accept other one carbon fragments (e.g. formimino group -CH=NH) and it is probable that the enzymes that are involved in its metabolism are not individual proteins, but exist as a multiple functioning complex.

Folic acid is absorbed into the body in its monoglutamate form and passes into the blood after methylation (Perry and Chanarin 1970). It is activated by being reduced at the 5,6,7 and 8 positions on its pteridine ring to tetrahydrofolic acid (THF) by dihydrofolic acid reductase (Burchall and Hitching 1965). Normally, there is only a low concentration of folic acid present because it is rapidly hydrogenated. Folate antagonist drugs in common use have a similar structure to the pyrimidine moiety of folic acid and are tightly bound to it (e.g. methotrexate for chemotherapy, trimethoprim for infections).

Folate polyglutamation

THF has different numbers of glutamate residues and these affect its properties. The monoglutamate acts as the transport form and the polyglutamate is found intracellularly and is metabolically active (Shane and Stokstad 1976). The monoglutamate is transported to the cell bound mainly to albumin (Soliman and Oleson 1976) and easily passes through cell membranes into cells where it is conjugated with extra glutamatic residues into the active polyglutamic form by folylpolyglutamate synthetase (FPS).

$$THF(Glu)_1 \text{ Glutamic acid} \rightarrow THF(Glu)_2 \ldots\ldots THF(Glu)_n$$

Polyglutamic folate cannot cross cell membranes and accumulates - a cellular folate concentration mechanism (Foo and Shane 1982). Conjugation is essential for cell survival. Mutant Chinese Hamster ovarian cells containing no conjugating enzyme required methionine, purines and thymidine to grow. Folate intracellular concentration decreased by 90% and no polyglutamate was produced. Wild cells having the enzyme functioned normally and had no special growth requirements. Hence this enzyme is vital not only for cellular retention, but also for activation and utilisation of folate (Taylor and others 1977). The actual number of residues on the polyglutamate varies between species, but appears to be from 5 to 10.

Foo and others (1982) showed that FPS has an

increased affinity with a decreased substrate potential for the higher conjugated folates; a type of end product inhibition. Folate cellular concentration will only increase up to a certain amount.

Taylor and others (1977) showed that in vitro FPS was unaffected by vitamin B_{12} deficiency although rats exposed to 50% N_2O for 24 hours made no polyglutamated THF. Addition of parenteral folic acid or 5 methylTHF did increase its production slightly whereas 5 or 10 N formylTHF or 5,10 methyleneTHF allowed its normal synthesis (Perry and others 1979). Chanarin and others (1980) suggested that formylTHF was the most active substrate for FPS and that active vitamin B_{12} was needed to formylate THF.

Other work has demonstrated that 5 methylTHF is a poor precursor for polyglutamate formation (Mcguire and others 1980, Cichowicz 1987). Perry and others (1980) showed that only 21% THF was converted into a polyglutamate form in rats. In contrast, McGuire and others (1980) using partially purified rat liver FPS showed much greater incorporation. Cichowicz (1985) using purified hog liver enzyme demonstrated that THF and 10 formylTHF were equally effective as substrates. As yet, the most effective substrate for FPS is still unknown.

Folate reactions

When converted into its active polyglutamate form, folate can undergo a variety of reactions (Fig.3).

a) FormylTHF

THF binds with formate at either its 5 or 10 N position and this reaction is catalysed by formylTHF synthetase (FTS). Formate is produced from many compounds (e.g. catabolism of methionine, sarcosine, glycine, serine, tryptophan, etc) and then can be oxidised to carbon dioxide or used by THF as a source of single carbon fragments (Friedman 1954). In rats, formate is oxidised to CO2 by THF dependent pathways (Eells and others 1982). N_2O inactivation of rat methionine synthase (MS) increased FTS activity suggesting how important this compound is to the body (Perry and others 1980).

FormylTHF may be oxidised to CO2 by formylTHF dehydrogenase and the substrate Km in pigs liver is 8.2 μM (Kutzbach and Stokstadt 1971). The concentration of celluler formylTHF is normally within this range and so a small increase in its concentration will result in a large increase in oxidation, a positive feedback system.

10 formylTHF is involved in the production of purines through 2 reactions (Smith and others 1982);

a) Carbon 2 - requires 5 amino 4 imidazole carboxamine transformylase (AICAR).

b) Carbon 8 - requires glycinamide ribonucleotide transformylase (GAR).

When rats were exposed to N_2O for 5 days, the activities of both enzymes were changed with that of liver GAR decreasing whilst for AICAR, it increased (Deacon and others 1983a). Their activity in rat marrow was less

altered (Deacon and others 1985).

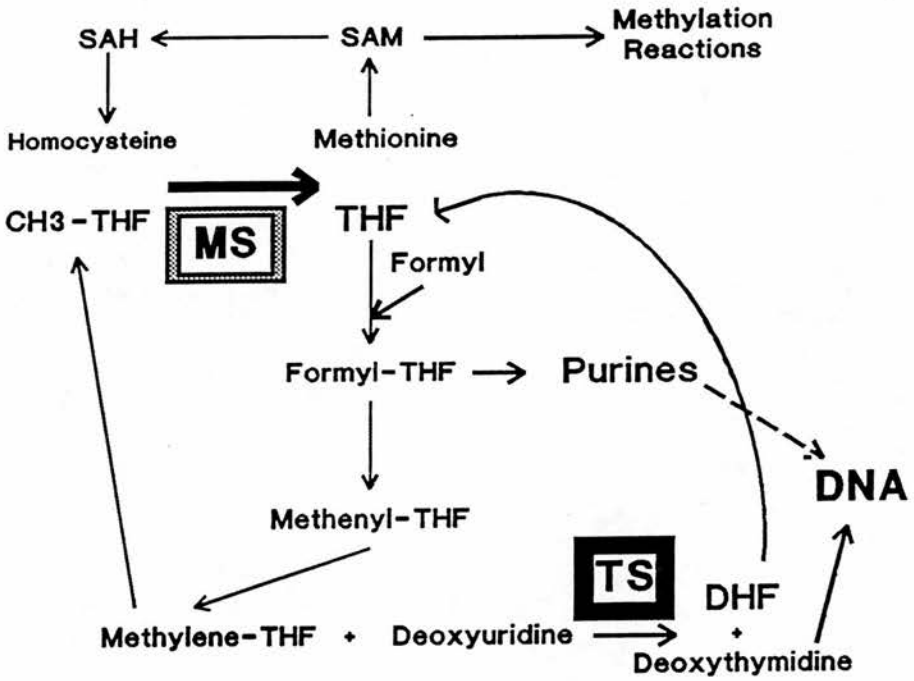


Figure 3. Tetrahydrofolate reactions. THF, tetrahydrofolate acid; DHF, dihydrofolic acid; DNA, deoxyribonucleic acid; SAM, S-adenosyl methionine, SAH, S-adenosyl homocysteine; MS, methionine synthase.

b) MethenylTHF

FormylTHF can be reduced to 5,10 N methenylTHF by the enzyme 5,10 N methenylTHF cyclohydrolase. Its activity in rats exposed to 50% N_2O was reduced (Perry and others 1980). Therefore, after N_2O inactivates vitamin B_{12}, the enzymatic response is to conserve formylTHF concentrations by increasing its synthesis and decreasing its catabolism.

c) MethyleneTHF

MethenylTHF is reduced by 5,10 N methenylTHF dehydrogenase to 5,10 N methyleneTHF. The activity of this enzyme is unchanged in rats exposed to N_2O (Perry and others 1980). 5,10 N methyleneTHF is involved in deoxythymidine monophosphate (dTMP) production because it supplies a methyl group to deoxyuridine monophosphate (dUMP). Thymidine synthetase (TS) catalyses this reaction and dihydrofolic acid which is produced is reduced back to THF by dihydrofolate reductase (O'Brien 1962). Sakamoto and others (1975) found that purified TS from marrow cells of patients with untreated pernicious anemia had a ninefold increase in activity. Deacon and others (1981a) showed a 200% increase in TS activity in N_2O exposed rats which returned to normal after the gas was removed. However, even though TS activity is increased, the dU test indicates that a decrease in deoxyuridine methylation occurs suggesting a shortage of 5,10 N methyleneTHF (Amess

and others 1978, Skacel and others 1982, Kano and others 1981, O'Sullivan and others 1981). It appears that N_2O inhibits folate metabolism by altering the concentrations of the different folate co-enzymes.

Humans have a greater dependence on vitamin B_{12}/folate pathway for TS synthesis of deoxythymidine than the rat which can use the salvage pathway to produce dTMP. Does this explain why this animal does not become megaloblastic when exposed to N_2O?

d) MethylTHF

5,10 N methyleneTHF can be further reduced to 5 methylTHF by 5,10 N methyleneTHF reductase. This reaction is thermodynamically a one way process becasue oxidation back into 5,10 methyleneTHF is unlikely <u>in vivo</u> (Kutzbach and others 1971). S adenosyl methionine (SAM), a non-competitive inhibitor of this enzyme, has a Ki=2.8 μM for pig liver (Kutzbach and Stokstad 1971). Normally SAM concentrations are less than this, but when they increase, there will be a profound inhibitory effect.

To complete the folate metabolic circle, THF is regenerated from 5 methylTHF by methylation of homocysteine into methionine. MS catalyses the transfer of the methyl group (Taylor and Weissbach 1973). SAM and a reducing system are initially needed <u>in vitro</u> to convert the inactive, but stable Co(II), into the active, but relatively unstable Co(I). Because this is the only reaction that 5 methylTHF undergoes, it is the only way

to regenerate THF. Patients with inherited defective MS have liver concentrations only 30% of normal and all have raised 5 methylTHF concentrations and clinical signs of megaloblastic anemia.

e) Other reactions

THF is involved in the metabolism of amino-acids. Serine is a major source of carbon units, its 3C being reversibly transferred to THF to produce 5, 10 N methyleneTHF and glycine.

Serine + THF ----> Glycine + 5,10 N methyleneTHF

There was no change in its activity in rats exposed to 50% N_2O for 15 days (Deacon and others 1980). THF is involved in histidine catabolism by accepting a formimino group from formiminoglutamic acid which is formed from histidine. This produces 5 formimino-THF and glutamic acid and is catalysed by 5 Formimino-THF transferase. The activity of this enzyme was unaffected in rats exposed to N_2O (Perry and others 1980).

Summary

THF can accept and transfer single carbon units to a variety of compounds. N_2O has effects on the different enzymes in its metabolic pathway (Table I); it fully inhibits MS and partially inhibits GAR and 5,10 N methenylTHF cyclohydrolase. It increases the activity of

FTS, AICAR and TS.

Table I. Effect of N_2O on the folate converting enzymes.

Enzyme	Affect
Folatepolyglutamate synthetase	0
FormylTHF synthetase	++
AICAR transformylase	+
GAR transformylase	-
MethenylTHF cyclohydrolase	-
MethyleneTHF dehydrogenase	0
MethyleneTHF reductase	0
Thymidylate synthase	++
Methionine synthase	---
Serine transhydroxymethylase	0
Formiminoglutamic acid transferase	0

+ = increase
- = decrease
0 = no effect

Folate co-enzyme levels

Secondary to its effects on folate enzymatic activities, N_2O can change the concentrations of the different folate co-enzymes. Lumb and others (1980) showed a decrease in totla liver folate concentration in N_2O exposed rats. Further work showed a loss of folate from other tissues including kidney, bone marrow and brain. Polyglutamates were affected more than monoglutamates and both the methyl and non-methylfolates were affected. Presumably, reductions in the rate of polyglutamation prevented the cells from retaining folate. They also discovered that there was a transient increase in tissue methylTHF concentrations and that total plasma folate was increased (Perry and others 1981). Additionally, N_2O exposed rats excreted large amounts of labelled 5 methylTHF without its methyl group being removed (Lumb et al 1982).

Rats exposed to 75% N_2O for 18 hours had the folate co-enzyme concentrations in the marrow, kidney, brain and liver measured (Wilson and Horne 1986). MS activity decreased by 90% in the marrow, kidney and brain and 83% in the liver. The proportion of methylTHF to non-methylTHF increased from 1.4 to 1.9 and there was a decrease in the concentrations of the non-methylTHF forms. No effect was seen on the distribution of the mitochondrial folate co-enzymes (Horne and others 1989). Similar changes in folate co-enzymes concentrations were seen in embryo rats whose mothers had been exposed to N_2O.

There was an increase in the methylated and a decrease in the non-methylated forms (Hanson and Billings 1985).

Hence, it appears that N_2O inactivation of vitamin B_{12} causes considerable changes in both the concentrations and type of folate co-enzyme present. Total tissue folate concentrations decline, mainly due to increased urine excretion and this is probably due to cells being unable to concentrate the monoglutamate form. Of the different co-enzymes, only the concentration of 5 methylTHF increases.

METHIONINE

Vitamin B_{12} and folate are intimately involved in methionine metabolism. Methionine, a sulphur containing amino-acid, is vital for protein synthesis and provides a variety of compounds, including one carbon fragments.

Methionine turnover is about 60 $mmol.day^{-1}$ of which about 10 mmol is ingested in the diet, 45 mmol is due to catabolism and protein synthesis and the rest used for transmethylation reactions (Nunn and others 1985).

Nitrous oxide effects on methionine concentrations

N_2O reduces methionine concentrations. Rats exposed to 50% N_2O for 24 hours had their plasma methionine concentration reduced by 36% (Lumb and others 1983) although in another study it by 82% (Vina and others

1986). Tissues are affected more, with liver and brain concentrations decreasing by 55% and 54% respectively after 24 hours (Vina and others 1986) and by 12% after 12 hours and 16% after 24 hours (Lumb and others 1983). With shorter exposure times of 80 minutes, the plasma concentration decrease was insignificant and in liver was only 38% (Royston and others 1989). Humans showed no decrease in plasma methionine concentrations during a N_2O anaesthetic lasting up to 217 minutes although with an 8 hour exposure it decreased by 30% (Skacel and others 1983). Pre-operative starvation decreased methionine concentrations (Nunn and others 1985).

Methionine metabolism

As well as protein synthesis, methionine has other important biochemical functions. The first step in its metabolism is the formation of a high energy compound, SAM. This then can either be a methyl donor or participate in the formation of polyamines.

Methionine + ATP ---> SAM + PP + P

The enzyme responsible is methionine adenosyl-transferase and 3 isoenzymes have been found in the rat, and are mainly in its liver.

I - (15%) Km met.=41 μM

II _ (5%) S0.5(met)=8 μM, inhibited by SAM,

found extrahepatically

III - (80%) S0.5(met)=215 μM, activated by SAM

The metabolic concentrations of SAM and methionine are 50-200 μM and 50-150 μM respectively, and hence the reaction will occur at maximum speed outside the liver. A negative feedback occurs when SAM concentrations increase. The liver enzyme is less active, but it will increase when SAM and methionine concentrations increase. Exposure of rats to N_2O for 80 minutes resulted in no changes in SAM concentrations in plasma, liver or brain (Royston and others 1989). This is in agreement with human data showing no decrease in plasma SAM concentrations after anaesthesia lasting 25 to 217 minutes (Nunn and others 1985).

Hence, in rats, even though thymidine synthesis is impaired after 60 minutes (Deacon and others 1980), SAM concentrations are normal. Therefore, it appears that this compound may not have any affect on how N_2O has its effects in the short term.

SAM is the methyl donor for many methylation reactions producing S-adenosyl homocysteine (SAH). The methyl group may be transferred to carbon, nitrogen or oxygen atoms (eg. synthesis of adrenaline, creatinine, etc). Most methyltransferases are inhibited by SAH, although the physiological importance of this path is unknown. A second pathway is in the formation of polyamines.

Ornithine -------> Putrescine

ornithine decarboxylase

SAM -------> dSAM

SAM decarboxylase

dSAM + Putrescine -------> Spermine/Spermidine + 5MTA

Spermine/Spermidine synthase

SAM decarboxylase is activated over the physiological range of putrescine concentrations (0-50 μM), so putrescine is efficiently converted to spermine. Because the lifespan of this enzyme is less than 2 hours, protein turnover regulates its activity and decreased spermidine increases protein formation. The supply of the dSAM probably regulates polyamine formation. The only route for further metabolism of dSAM is probably via the polyamine pathway. Whilst the function of polyamines is still unknown, it appears that they may be involved in the regulation of macromolecular synthesis (Grillo 1985) and this may be partially responsible for the decrease in DNA synthesis - as yet this possibility has still to be examined.

5MTA is then able to be recycled to form methionine.

5MTA ------> 5MTR,1,P

MTA phosphorylase

This is then isomerised to 5 methylthioribulose and converted to 1-keto 3-methiolbutyrate which can be transaminated to methionine. SAM is also an important

precursor of formate which is formed from methylthioribose (Trackman and Abeles 1981). Hence SAM is important and produces a variety of vital compounds. Because its concentrations remain constant during exposure to N_2O, changes in its biochemistry are probably not the cause of N_2O toxicity.

SAH can be hydrolysed to homocysteine by SAH hydrolase, a reversible reaction with an equilibrium in the direction of SAH formation. However, when adenosine and homocysteine are absent, hydrolysis occurs. Accumulation of these compounds will inhibit the formation of homocysteine.

Homocysteine is metabolised in several ways.

1) to methionine by MS
2) to methionine by the betaine pathway
3) to cystathionine
4) to SAH

Hence, it is an important regulatory point. Its conversion to methionine by MS has been discussed. The betaine pathway is only found in mammalian liver and is specific for homocystine. The Km for betaine and homocystine for the rat and human liver are 50 and 16 and 100 and 120 μM respectively.

Choline ----> Betaine + Homocysteine ------> Dimethyl Glycine + Methionine

B-H methyltransferase

The enzyme is inhibited <u>in vitro</u> by dimethyl glycine, methionine and SAM and is induced by a methionine deficiency and increases in homocysteine, betaine or

choline concentrations. Therefore the enzyme acts to remove excess substrate. This pathway may explain why rats do not have depressed marrows on chronic exposure to N_2O because methionine concentrations are maintained. However, it will have no effect on the distribution of THF co-enzymes.

Alternatively, homocysteine can be catabolised by the transsulfication pathway.

Homocysteine + Serine -------> Cystathionine

Cystathionine Synthase (CS)

CS Km for homocysteine and serine is 0.7 mM and 0.1-0.4 mM respectively and SAM activates it. Approximately 27% of homocysteine in rats is metabolised by both the MS and betaine pathways and CS removes the rest.

Methionine has a regulatory role over folate metabolism. Isolated, methionine depleted liver cells have normal MS activity, but cannot metabolise histidine or formate (Krebs and others 1976). This is similar to rats exposed to N_2O with inactivated MS (Eells and others 1982). Methionine reversed this. How methionine has its regulatory effects is unknown although it may be via SAM.

MECHANISMS OF NITROUS OXIDE TOXICITY

It is agreed that N_2O causes marrow depression by decreasing DNA synthesis because of abnormal folate metabolism. The dU test demonstrates that dTMP production decreases. Because this compound is usually phosphorylated

to dTTP and incorporated into DNA, its shortage will decrease DNA synthesis and reduce cell replication. The abnormal dU test is caused by a decrease in 5,10 N methyleneTHF concentration. Some 5,10 N methyleneTHF is produced from serine, although this pathway is unimportant in humans. There may also be a decrease in purine production due to a reduction in 5 formylTHF concentrations. Because purines are needed for DNA synthesis, a decrease in their concentration will further inhibit DNA formation. Purines are also required for RNA formation and protein synthesis. The effect of N_2O on these compounds have not yet been fully investigated although pregnant rats exposed to 50% N_2O for 24 hours on day 10 had a decrease in both DNA and RNA fetal content whilst the amount of cellular purine remained normal (Hansen and Grafton 1988). Perhaps decreased polyamine synthesis was responsible.

However, how MS inhibition by N_2O causes an abnormal folate metabolism is uncertain. Whilst many theories have been proposed, the two most popular are the methyl trap and the formate starvation hypothesis.

The Methyl Trap Hypothesis

Herbert and Zalusky (1962) and Noronha and Silverman (1962) proposed the methyl trap hypothesis. They used experimental evidence showing that the only common link between folate, methionine and vitamin B_{12} metabolism is MS and that in vitamin B_{12} deficient rats and humans there

was an increase in the amount of folate existing as 5 methylTHF. They suggested that after MS was inhibited there would be a "trapping" of folate in its 5 methylTHF form. Eventually most would become methylated resulting in a functional deficit of the other co-enzymes leading to an impairment of its reactions. Consequently there would be a decrease in thymidine and purine biosynthesis with decreased DNA formation.

Much evidence supports this hypothesis. Many studies show that if vitamin B_{12} deficiency occurs, there is an increase in the 5 methylTHF portion of the folate pool (Shane and Stokstad 1985, Perry and others 1976, Wilson and Horne 1986, Horne and others 1989). It is unlikely that 5 methylTHF is metabolised by any other pathways except through MS as thermodynamically its formation from 5,10 N methyleneTHF is so favourable. However, work has shown that under certain conditions, 5 methylTHF can be oxidised back into 5,10 N methyleneTHF. In vitro lymphocytes from a patient with pernicious anemia were shown to oxidise 5 methylTHF (Thorndike and Beck 1977), although, unphysiological conditions were required. Fuller (1976) reviewed the possibility of direct methylation of biogenic amines by 5 methylTHF and he suggested that whilst it can occur unphysiological conditions are required. Hence, in humans there is no evidence for a separate vitamin B_{12} independent pathway that can metabolise 5 methylTHF.

Methionine prevents the clinical effects of vitamin B_{12} deficiency (Noronha and Silverman 1962, Brody and

others 1982) and this, according to the theory, is because methionine is the precursor of SAM and increased methionine concentrations will increase those of SAM. This compound inhibits 5,10 N methyleneTHF reductase and this will eventually reduce the amount of folate converted to 5 methylTHF and thereby prevent its trapping.

This explains how methionine preserves THF concentrations, but not how it restores it when it is already "trapped". There is evidence that the rat can escape from the methyl trap. Rats were given a mixture of ^{14}C 5 methylTHF and 5 methyl-^{3}H-THF and with air the methyl group had a half life of 2 hrs. After exposure to N_2O for 78 hours, 40% remained as methylTHF, 39% was the polyglutamated and 20% was found on non-folate compounds (Lumb and others 1984). Methionine has rapid effects on folate concentrations and the inhibitory effects of SAM may not be responsible for the rapid changes caused by methionine.

There is other evidence which is difficult to explain by the methyl trap hypothesis. Deacon and others (1980) found that formylTHF could correct abnormal dU suppression tests in vitamin B_{12} deficient patients whilst THF was unable to although if the methyl trap hypothesis is correct, it should do. THF added to marrow cells from vitamin B_{12} and/or folate deficient patients was not metabolised as expected (Deacon and others 1981). Takeri and others (1982) examined marrows from similar patients and found that THF partially corrected the abnormality although not as effectively as formylTHF. However,

formylTHF is more stable than THF and in folate deficient cells, formylTHF was more active than THF in correcting abnormalities. Perhaps formylTHF may enter the cell more efficiently than THF.

The Formate Starvation Hypothesis

Chanarin and others (1980) examined rats exposed to N_2O and proposed a new theory to explain how 5 formylTHF prevented the effects of N_2O whilst THF did not. They realised that methionine increased folate polyglutamate concentrations in the liver (Smith and others 1974) and it reversed the impaired synthesis in N_2O exposed rats (Perry and others 1983). SAM is equally effective at a slightly higher concentration, although it is only partially able to cross cell membranes (Zappia et al 1978). Even more effective is 5 methyl-thioadenosine (Perry and others 1983) and methionine produces formate via 5 methyl-thioadenosine. Folate concentration decline when methionine concentrations are low. Normally, up to 87% of the methionine can be converted to either formate or to CO2 (Case and others 1977).

Formate is used as either a donor of one carbon fragments or is oxidised to CO2. Both these reactions occur via folate co-enzymes (Friedman and others 1954). Labelled formate was oxidised to CO2 and this occurred 50% slower in N_2O exposed rats (Eells and others 1981). There is an increase in activity of formylTHF synthase and a decrease in cyclohydrolase after N_2O exposure

suggesting that the body conserves formylTHF (Perry and others 1980).

The hypothesis states that the prime defect of cobalamin inactivation is a failure to supply methionine due to a decrease in its production after MS inhibition. Methionine produces formate and the main substrate for cellular retention and activity of the folate co-enzymes is 5 formylTHF. Therefore a decrease in formate concentrations will cause a decrease in this folate co-enzyme.

Methionine in this theory would be expected to overcome the decreased formate supply and experimentally this is true (Skin and others 1975, Scott and others 1980, Krebs and others 1976). Additionally, it explains the effectiveness of folinic acid (5 N formylTHF) in preventing megaloblastosis after N_2O exposure (Amos and others 1984).

However, it was shown that isolated purified FPS could use THF as a substrate equally as well as formylTHF and only folic acid and 5 methylTHF were poor (Moran and others 1980, Cichowic and others 1987). The methyl trap hypothesis could explain the above data as 5 methylTHF is a poor substrate for FPS. Additionally formylTHF may be more stable than THF and experimental errors may have occurred. Additionally, it is possible that formylTHF can enter cells more rapidly than THF to produce higher concentrations.

Further evidence indicates that there is no decrease in plasma or brain SAM or methionine concentrations in

rats exposed to N_2O for up to 80 minutes. A decrease in methionine concentrations was seen in the liver although SAM concentrations remained stable. Therefore it is unlikely that decreases in methionine and SAM concentrations are responsible, at least in the early stages, for the effects on DNA synthesis.

Conclusion

Folate and the co-enzymes and enzymes that are involved in its metabolism have been described. The effects that N_2O has on these have been illustrated. There is a decrease in total tissue folate concentrations with a relative increase in that of 5 methylTHF compared to the other co-enzymes. This probably results in an general reduction in the formation of deoxythymidine and therefore a decrease in cellular DNA synthesis. Methionine metabolism is important for cellular activity. The evidence for and against the methylfolate trap and the formate starvation hypotheses tends to suggest that the former may be responsible for the abnormal folate metabolism.

CHAPTER THREE

THE CLINICAL EFFECTS OF THE REACTION OF NITROUS OXIDE WITH METHIONINE SYNTHASE

Introduction

Methionine synthase (MS) inhibition by N_2O causes a reduction in DNA synthesis throughout the body. This may be of no clinical significance, although it has the potential to cause serious problems especially after prolonged exposure. In addition, it is uncertain whether other, more subtle effects also occur and whether they are exacerbated by the effects of anaesthesia and surgery.

The evidence for these possibilities is presented in this chapter by examination of the clinical and biochemical studies performed on N_2O. Epidemiological evidence will also be assessed.

Exposure to N_2O

Humans are exposed to N_2O in two ways, acutely in high concentrations usually over a short time when given for anaesthesia or pain relief and chronically, to trace concentrations over a longer time. Both types of exposure may have clinical effects. Because toxicity is dose dependent, a simple method for comparing different exposures has been developed;

Concentration of N_2O (atm) x duration of exposure (h)

An acute exposure dose is easily found if both the concentration and duration of N_2O given is known. However, measurement of the amount of exposure to N_2O that occurs chronically is more complex because it is affected by several factors. Many methods have been developed to

measure it, although all have two basic criteria. The air must be sampled and then its N_2O concentration measured. Sampling can be active or passive. Active methods involve suctioning air into a storage or analytical system while passive systems allow gas to diffuse into small samplers packed with a molecular sieve. Passive methods allows N_2O concentrations to be assessed over a longer time (Gray and others 1986). Collection is either continuous or intermittent ("grab"). Because concentrations vary over time, grab sampling is inaccurate unless there are multiple samples collections with the results being averaged. Continuous sampling can be used to calculate a time-averaged concentration and this is more accurate a measurement for longer durations. Sampling may be from a person's breathing space (personal sampling) or from the work area (area sampling) with the former measuring individually inhaled gas concentrations. In contrast, area sampling assesses overall theatre contamination. Unfortunately gas concentrations vary throughout theatre. Using an infrared camera, N_2O clouds could be seen in some parts of the theatre with others having little contamination (Allander and others 1981). Area sampling is a poor index of a person's inhalation.

People exposed to N_2O include theatre workers (anaesthetists, surgeons, nurses, etc), dentists (and dental assistants) and vets. Pollution in these cases results from inadvertent leaks of anaesthetic gases from the anaesthetic machine and from exhalation by patients. Midwives and ambulance workers are exposed through the use

of Entonox as an analgesic.

Levels of N_2O theatre contamination

Davenport and others (1980) measured N_2O and halothane contamination over a 2 hour period in the operating theatres, anaesthetic areas and recovery rooms of 20 hospitals. They used active integrated area samplers which collect theatre air at a constant flow rate by active suction. N_2O contamination varied from <10 to 3000 ppm and averaged 388.5 ppm. Because pollution in operating areas can fluctuate, air was sampled from in front of the anaesthetist's nose. No relationship between N_2O concentrations from here and ambient air was found.

Gray and Spence (1984) analysed waste anaesthetic gas pollution on 5 successive days in 2 operating theatres. One contained a passive and the other an active scavenging system. Personal pollution was measured using the Casella evacuated canister system which was positioned on the individual's lapel. In the passively scavenged theatre the anaesthetists N_2O exposure ranged from 40 to 700 ppm while it was less in the actively scavenged theatre (30 to 200 ppm). Anaesthetists were exposed to greater amounts of N_2O than nurses or other theatre assistants.

Gray (1989) studied N_2O exposure of anaesthetists, anaesthetic assistants and "circulating" nurses in every operating theatre in Glasgow. A metal tube sampler packed with a molecular sieve that absorbs N_2O passively by diffusion was used and the gas concentrations were

measured by gas chromatography (Gray and others 1986). The samplers were attached to the tunic near the subject's breathing zone and a 1-2 week period of exposure was measured. Anaesthetists exposure was greater than 100 ppm for more than 50% of the time.

These concentrations can be compared with suggested maximum values from the National Institute of Occupational Safety and Health (NIOSH); 25 ppm N_2O and 0.5 ppm halothane. In Sweden, the recommended 8 hour time averaged exposure is 100 ppm (Sik and others 1990). In contrast, the Department of Health and Social Security (DHSS) recommendation is that the limit should be as low as possible which allows individual Health and Safety Executive inspectors to make a local interpretation (DHSS 1976).

Hillman and others (1981) assessed pollution of a passively ventilated dental surgery over a 6 month period. Sampling was active, 6-8 inches from the dentist's nose and continuous N_2O concentrations were found using an infrared monitor. The concentrations were;

a) without scavenging 266-6281 (mean 2445) ppm
b) with scavenging 92-1228 (mean 351) ppm

Other work has found similarly high amounts of pollution (when no scavenging is used) ranging from 350-8500 ppm (Campbell and others 1977, Whitcher and others 1977). Blood N_2O concentrations are theoretically more accurate in assessing personal contamination and they indicate that dental workers inhale more N_2O than theatre

workers. Hillman and others (1981) measured blood N_2O concentrations in both dentists (mean 60.3, SD 31.4 μmol $N_2O.litre^{-1}$) and dental assistants (mean 20.3, SD 8.5 μmol $N_2O.litre^{-1}$) in non-scavenged rooms and found they were considerably greater than those in anaesthetists who worked in non-scavenged theatres (14.9 μmol N_2O/litre)(Krapez and others 1980).

Munley and others (1986) studied midwife N_2O exposure over a shift in 4 maternity hospitals using personal sampling. In 3 hospitals, mean exposure was less than 100 ppm, but in the 4th, it was greater than 360 ppm and scavenging reduced this to 150 ppm. The main reasons for these differences appear to be different working practices and the layout, size and ventilation of the labour suites. lsley and others (1989) examined the duration and frequency of Entonox administration inside ambulances. N_2O concentrations were found using grab samples at one minute intervals from the breathing zone of both the attendant and driver whenever Entonox was used. Mean exposure time for attendants was 11.4 (13.8) minutes to mean concentrations of 1100 (3900) ppm with open (closed) windows. The driver's exposure was half this and the highest exposure of any individual was 56,000 ppm.

Hence, many types of worker are exposed to N_2O with the actual duration and concentration of exposure varying greatly. Overall, dentists have the highest exposure, presumably due to their particular type of anaesthetic practice (high flows, leakage from mask, poor scavenging, etc). Anaesthetists and other theatre workers are exposed

to high concentrations especially in unscavenged theatres.

Human exposure and MS activity

Patients undergoing anaesthesia are exposed to N_2O in concentrations which are high enough to inhibit MS. However there is less known about the effects of trace concentrations. Work on animals exposed to trace concentrations has been discussed previously, but in summary, the ED_{50} for liver MS inhibition in rats after 1 days exposure was 10,400 ppm and after 2-28 days was 5,400 ppm. The lowest N_2O concentration to affect MS was 840 ppm for 1 day and 450 ppm for 48 hours (Sharer and others 1983). Koblin and others (1981) showed that exposure to less than 50,000 ppm N_2O for 4 hours had no effect on mice MS. Extrapolation indicates that MS is 50% inactivated after 4 hours exposure to 10% N_2O. Exposure to 1100 ppm for 8 to 22 days caused a small, but significant decrease in MS activity and recovery took 2 to 4 days. Therefore, in rodents, MS is inhibited by exposure to trace concentrations of N_2O. Is this clinically relevant to human workers because human MS appears more resistant to inhibition (Royston and others 1989)?

Cell division and the tissues involved

When DNA synthesis decreases, all cells in the body are affected although those requiring large amounts of DNA (i.e. the rapidly dividing tissues) will be the first to

be affected. Organs and cells tested for abnormalities after N_2O exposure include the bone marrow, the immune system, the fetus and cells involved in wound healing and spermatogenesis.

Because N_2O impairs vitamin B_{12} and folate metabolism, patients with abnormalities in either of these vitamins may be especially susceptible to N_2O. However, in a study of chronic alcoholics (who tend to have a folate deficiency) exposed to N_2O for 20-60 minutes, no megaloblastic changes were seen in their peripheral blood films, although this is a crude measure of diagnosing early marrow changes (Editorial 1987c). Two women developed neurological complications similar to those of subacute combined degeneration of the cord within 8 weeks of anaesthesia. One had had pernicious anaemia and the other a previous ileal resection for Crohns disease (Schilling 1986). In animals, synergy between vitamin B_{12} deficiency and N_2O exposure has been shown (O'Leary and others 1984). Hence, it is possible that patients with vitamin B_{12} deficiency may be particularly susceptible to N_2O toxicity (eg Vegans, pernicious anaemia and malabsorption diseases, etc). Folate deficiency is relatively common and can occur from poor diets, pregnancy and malabsorption. Some chemotherapeutic agents interfere with folate metabolism. For example, methotrexate inhibits dihydrofolate reductase and this may theoretically cause increased toxicity (Ueland and others 1986).

1. Cell Division

N_2O is known to interfere with cell division (Brinkley and Rao 1973). All anaesthetic drugs cause a replicating cell to remain in mitosis by a non-specific general action. The ED_{50} of halothane's that stops the replication of the root of the broad bean in the metaphase of mitosis is 0.5 - 0.9% (Nunn and others 1971). Other anaesthetic drugs caused a dose dependent reduction in mammalian Chinese hamster cell replication by inhibiting every phase of the cell cycle although N_2O was not tested (Sturrock and Nunn 1975). However, 75% N_2O had similar effects to 0.5% halothane in suppressing the division of cultured murine bone marrow cells (Nunn and others 1976), but this is probably not a specific effect.

2. Mutagenicity and Carcinogenicity

N_2O may be both mutagenic and carcinogenic due to its effects on DNA synthesis. Because carcinogens are always mutagens (Duncan and Brookes 1973), mutagenicity is an essential test for any carcinogenic chemical. It is a simpler test to perform because the standard bioassay for carcinogenicity involves lifelong exposure of large number of animals to the drug. The mutagenicity of N_2O has been extensively investigated using a variety of tests. Sturroch (1977) examined the effects of halothane both with and without N_2O on Chinese Hamster lung fibroblasts

using the 8-azaguanine assay system. Hypoxanthineguanine phosphoribyl transferase converts guanine and hypoxanthine to guanylic and inosinic acids. Fibroblasts are capable of absorbing 8-azoguanine from a culture medium and if no guanine is present, they incorporate it into their DNA and RNA and the cell will die. However, if the cell is a mutant lacking this enzyme, it survives because it can make guanine in another way and a colony forms. Exposure of these cells to 75% N_2O with 1-3% halothane for 24 hours resulted in no colony formation and hence no significant mutation had occurred.

White and others (1979) examined the frequency of sister chromatid exchanges (SCE) in Chinese Hamster ovarian cells. This is a rapid <u>in vivo</u> assay of genetic damage because mutagens will increase the rate of SCE during cell division. Exposure to 1 MAC N_2O for 1 hour had no effect. Baden and others (1979) used the salmonella microsome assay of Ames and others (1975). Two histidine dependent strains of S.typhimurium, both of which can revert either spontaneously or by mutagenic change to independency, were exposed to 1-81% N_2O for 8 hours in agar. No mutagenic changes occurred and further work (Baden and others 1981) exposing similar bacteria to high pressures of N_2O had identical results.

Baden and Kundomal (1986) exposed male Drosophilia flies to various concentrations of N_2O and halothane, enflurane or isoflurane for 1 hour and showed that N_2O was not an enhancer of the mutagenic properties of these volatile anaesthetics.

Not all tests have been negative. Exposure of Drosophilia flies to 100% N_2O for 6 minutes resulted in increased mutagenicity (Garrett and Fuerst 1974) although this may have been due to hypoxia. A second test has shown N_2O to be weakly mutagenic, although it used a less reliable test which examines the colour of stamen hairs in Tradescantia.

Overall, it appears that N_2O is not a mutagen or at worst, is a very weak one. Therefore, it is highly unlikely that it will be a carcinogen. To examine this 2 studies have been carried out. Eger and others (1978) exposed male and female Swiss ICR mice to 19-75% N_2O for 2 hours each day at 2 day intervals during the last half of pregnancy and for 24 times at 2-3 day intervals 5 days after birth. The mice were killed at 9 or 15 months. There was no evidence of an increase in the incidence of tumours. Coate and others (1979) studied the effect of low concentrations of halothane and N_2O on male and female Fischer rat's reticulo-endothelial systems. Exposing them to either 1 or 10 ppm halothane with 50 or 500 ppm N_2O for 7 hours a day, 5 days a week for 104 weeks caused no increase in malignancy. Overall, there is no evidence that N_2O has any mutagenic and carcinogenic properties.

Many epidemiological studies have studied the effect waste anaesthetic gases have on the morbidity or mortality of exposed individuals. Unfortunately, most of the studies have an intrinsic problem in how they are designed because of unsuitable control groups. Unfortunately there are many

factors in the theatre environment that could potentially influence the health of these workers and so do these studies reflect the effects of anaesthetic gas exposure alone? Working in theatres can be stressful and this may be partially responsible for any increases in spontaneous abortions that have been seen (Fink and Cullen 1976). There is also an increase in exposure to other potentially lethal agents and possible biological agents include organisms in blood (eg hepatitis B, HIV virus). Chemical agents unique to theatre may be toxic (methylmethacrylate) and in some theatres there is an increase in radiation exposure. Exclusion of these possibilities requires a study design which uses closely parallelled control groups who have similar work conditions, but are not exposed to anaesthetic gases. Therefore most studies have examined the effect of working in a theatre rather than of exposure to anaesthetic agents as the cause of morbidity or mortality.

There are other problems in the design of these studies. In the exposed groups there is little or no attempt to quantify the amounts and types of anaesthetic gas exposure. Some individuals may have had a heavy exposure to N_2O with little inhalation of halothane whilst others from the same group have completely different patterns of exposure. One theatre nurse may work exclusively in the recovery room with little anaesthetic gas exposure and another assists the anaesthetist throughout the operation. The groups therefore all have different gas exposures. Additionally the amount of N_2O

present in theatre air can vary greatly.

There are problems in data collection because most studies are retrospective and use postal surveys in which individuals are asked to report any relevant past medical history. This form of enquiry has many problems (Spence 1987). Recall of past medical illnesses can be inaccurate. Some individuals are more motivated than others to respond especially if they realise that the study affects them personally and this can reduce the control group response rate. For example 70% of an exposed group and 41% of the control group responded in the 1974 ASA survey. Different motivations may cause variations in the accuracy of recall of problems. Verification of the response data is difficult and it is often impossible to prove the accuracy of the data. It is appreciated that surveys and interviews are inaccurate when compared to data collected from medical records (Lilienfeld and Lilienfeld 1980). However, there have been a few studies in which registers have been used to overcome this problem (Ericson and Källen 1979, 1985, Hemminki and others 1985).

Hence the conclusions of these studies is questionable. Four studies have looked at the morbidity of cancer (Corbett and others 1973, ASA 1974, Tomlin 1979 and Cohen 1980) and five have examined the mortality from cancer (Bruce and others 1968, Bruce and others 1974, Doll and Peto 1977, Lew 1979 and Neil and others 1987).

Corbett and others (1973) surveyed 621 female nurse-anaesthetists with a response rate of 84.5% (525 responses) and found 33 malignancies in 31 nurses. The

control group was based on statistics from the Connecticut Tumour Registry with an expected tumour rate of 402.8/100,000 compared to 1,333/100,000 in the nurse group. The ASA (1974) published a national study of occupational disease in theatre personnel using a postal survey of 49,585 exposed individuals and 23,911 non-exposed individuals as controls. The incidence of tumour increased only in females (from 130% to nearly 200%) and statistically, only leukaemias and lymphomas showed a significant increase ($p=0.05$). Cohen and others (1980) looked at tumour incidence in 30,650 dentists and 30,547 female assistants. They were divided into groups who used and did not use anaesthetic gases and then further subdivided into groups where N_2O alone (81.3%) or with other anaesthetics (19%) was used. Therefore, the specific effects of N_2O could be found. Male dentists were unaffected whilst female assistants had a slight, but insignificant increase in tumour incidence.

The five surveys that have examined anaesthetist's mortality have been mainly negative. Bruce and others (1968) found that 17 anaesthetists had died from tumours of the lymphoid and reticular-endothelial systems as compared to an expected 8.9 ($p=0.05$) although his second survey failed to confirm this (Bruce and others 1974). Doll and Peto (1977) studied 20,540 male British doctors who were older than 35 in 1951 for 20 years. Their occupations were found from the Medical Directory in 1952 with 547 and 704 being full and part time anaesthetists respectively. There was no increase in mortality although

slightly more died from pancreatic cancer than was expected. Lew (1979) examined mortality in members of the American Society of Anesthesiologists from 1954 to 1976. Death certificates from 610 male and 60 female anaesthetists (out of 637 and 66 deaths respectively) indicated no increase in the expected incidence of cancer when compared to controls. Neil and others (1987) followed up 3769 male anaesthetists in the UK between 1957 and 1983 and 221 died during this period. The control group consisted of social class 1 males and the total standardised mortality ratio (ratio of observed to expected deaths expressed as a percentage) was 68% (95% confidence of 59-77%) and for cancer, it was 50% (36-67%). There was no increase in the incidence of leukaemias or lymphomas although there was a greater incidence of suicide (ratio 202%, with 95% CI 115-328%).

Overall, it appears that there is little epidemiological evidence for carcinogenicity or mortality after exposure to anaesthetic gases. Expert analysis of these studies was performed using the relative risk (RR) measurement to find any possible association between exposure and disease.

$$RR = \frac{\text{Rate of disease among exposed individuals}}{\text{Rate of disease among unexposed individuals}}$$

The results of each study were pooled and the RR assessed. When it is greater than 1.0 the association is likely be true and when less, false. The higher the RR, the greater the association. Only studies with adequate

size and control groups were included and an overall RR for malignancies for men was 1.07 and women 1.4. Hence it is possible that women show an increased risk of malignancy when working in theatres although the association is only marginal (Buring and others 1985).

3. Bone Marrow

N_2O causes bone marrow depression. Investigations used to assess this have included examining peripheral blood pictures, direct visualisation of bone marrow and the dU suppression test. The evidence shows that prolonged N_2O exposure results in megaloblastic marrow changes whilst vitamin B_{12} concentrations remain normal. Since Amess and others published their study in 1978, there has been much work which has examined this effect. The exposure needed to cause depression, its duration, the effect of intermittent exposure, the effect on ill people and methods of preventing it have all been assessed.

Skacel and others (1983) anaesthetised 9 patients with 70% N_2O; 5 for 24 hours, 1 for 36 hours and 3 for 4-12 hours. The other 3 patients were controls and received etomidate. All except one individual underwent aortic surgery. Their peripheral blood films were assessed daily and amino-acids and folate concentrations measured 4 hourly. Bone marrow was examined by microscope and with the dU test pre-operatively, 24 hours later, on the 3rd or 4th day and after 1 week. Platelet numbers decreased in all patients for 3 days. The neutrophil count initially

increased and then decreased in the N_2O group after 24 hours (especially in 2 patients, one who received 36 hours of N_2O and the other, an alcoholic who received N_2O for 6 hours). All N_2O exposed patients had an increase in hypersegmented neutrophils counts on the 5th day which peaked at 7-9 days. After 24 hours, all 24 hour N_2O exposed patients had grossly megaloblastic marrows although they had improved after 3 days and were normal by 1 week. The marrows of control patients and the 3 who received only a small amount of N_2O were normal throughout. Similar results were seen with the dU test. Folate analogues were added <u>in vitro</u> to marrow samples and 5 formylTHF reversed the changes most effectively, although tetrahydrofolic acid (THF) and vitamin B_{12} also worked. In contrast, 5 methylTHF had only a limited effect. Serum folate concentration increased during N_2O inhalation while methionine concentration decreased in both groups, although this was greater in the N_2O group and continued throughout the inhalational period. Vitamin B_{12} concentrations were unchanged.

Hence it took at least 48 hours for marrow changes to recover. Are these changes important or do they have no clinical relevance? Because the bone marrow changes affected both leucocyte numbers and morphology, they must be important. The decrease in methionine concentrations could have adverse effects on protein synthesis or on methionine's other functions. One patient who appeared to be especially sensitive to N_2O was a chronic alcoholic who was exposed to N_2O for only 6 hours. Chronic ethanol

ingestion is recognised to disrupt folate metabolism (Barak and others 1987) and it is possible that N_2O aggravates this although acute doses of ethanol had no effect on the rate of mice MS inactivation by N_2O and this suggests no interaction between N_2O and acute ethanol intake (Tomerson and Koblin 1988).

Amos and others (1982) studied acute bone marrow changes and abnormalities in DNA synthesis in 70 seriously ill patients who were admitted to an intensive care unit for at least 24 hours. Any who had received folic acid supplements were excluded. There were 50 surgical and 20 medical (control) patients. All the surgical patients had a 1-6 hour anaesthetic although the N_2O concentrations used were not specified.

Twenty two patients had megaloblastic marrows on admission, 18 having had received a minimum of 2 hours N_2O within the previous 24 hours. Patients who had received N_2O had a greater chance of developing bone marrow abnormalities if illness was more severe (mean APACHE score of 31 compared to 19 in those patients with no changes). Sixteen N_2O expsoed patients died compared to 7 of the controls. Thirty-nine out of the 42 patients who received N_2O had an abnormal dU test and its value was proportional to the duration of N_2O exposure. The dU test correction pattern was typical of that in B_{12} deficiency in 30 patients. Normal dU tests were seen in all control patients. Eleven of the 22 patients with a megaloblastic bone marrow on admission were still in the ICU 3 days later. Three with a medical problem had normal marrows,

but 4 of the other 8 surgical patients had megaloblastic marrows when examined microscopically. All these dU tests were abnormal. Hence, it appears that admission of seriously ill patients to an ICU after a N_2O anaesthetic is more likely to be associated with megaloblastic marrow. The abnormalities were not only more severe, but also persisted for longer and in some patients became worse.

Further work by the same group (Amos and others 1985) tried to find a cause for the persistent megaloblastic changes. They studied 48 patients admitted to ICU for up to 15 days, 36 having had surgery (0.5-6 hours N_2O exposure). N_2O concentrations and APACHE scores were given. Sixteen (33%) had megaloblastic marrows when admitted of which 13 had been anaesthetised with N_2O. After 3 days, 9 were still megaloblastic (mean exposure to N_2O [3.7 hours] was greater than in those without [3.2 hours]). All patients not exposed to N_2O had normal dU tests, but 26 patients from the exposed group had abnormal tests and this decreased to 22 after 3 days. An additional 7 became abnormal. At admission, the abnormal dU test was corrected by both methylcobalamin and folic acid in 10 out of 12 samples and after 3 days in 4 out of 10. The rest were corrected by folic acid alone. Serum folate concentrations were low after 3 days in the N_2O exposed patients although there was increased urinary folate excretion in the non-N_2O exposed group. High doses of folic acid (15 mg IV daily) prevented abnormal dU tests occurring in 3 patients whereas low doses (400 μg IM daily) had no effect in 5 patients.

These results support previous findings which showed that ill patients exposed to N_2O have an increase in the incidence of abnormal dU tests and this appears due to folate deficiency. However, because there was no increase in folate loss in the urine, the actual mechanism of this deficiency is unknown.

Why severely ill patients should be more susceptible to N_2O is unknown. Perhaps it is because the illness reduces the body's reserve to cope with abnormalities. Other circumstantial evidence supports the possibility. A case report described a severely ill patient who had been stabbed and who received 70% N_2O for 105 minutes. He needed 31 units of blood and after 7 hours had a dU value of 18.4%. Severely ill patients with tetanus have been shown to have marrow depression and many have died after N_2O. This is in contrast to patients who receive it for pain relief (myocardial infarction, etc). However, caution is needed in interpreting these results because megaloblastic haematopoiesis has been noticed in severely ill patients who are not exposed to N_2O (Ibbotson and others 1975).

The amount of N_2O exposure needed before megaloblastosis occurs is variable. Nunn and others (1982) described a patient who used Entonox for physiotherapy after an attack of acute intermittent porphyria. He was given it for 15-20 minutes periods 3 times a day. After 24 days (total N_2O exposure of 18 hours) his marrow was megaloblastic with a dU test of 14.5% and addition of

vitamin B_{12} reduced it. N_2O was stopped and 7 days latter, he had a normal marrow and dU test. N_2O was recommenced at 20 minutes twice a day, but after 2 weeks his marrow was again megaloblastic although the dU test was normal. Recovery of marrow activity is slow, taking over 48 hours and this intermittent chronic inhibition is interesting because it appears that the effects are cumulative.

Attempts have been made to prevent marrow depression occurring. Reversal of abnormal dU tests in vitamin B_{12} or folate deficient patients with 5 formylTHF (folinic acid) have been successful (Metz and others 1968, Das and others 1970, Van der Weyden 1973). Folinic acid prevents increases in dU test results in animals (Deacon and others 1980). O'Sullivan and others (1981) studied the efficacy of folinic acid in preventing the development of a megablastic marrow. Thirty patients received a 1-6 hour anaesthetic with N_2O for routine surgery and a second group of 24 patients underwent cardiac surgery. Five received 50% N_2O for 12 hours, 6 for 24 hours and 7 for 24 hours. The last group were given 20 mg folinic acid orally, 12 and 1 hour before anaesthesia. The remaining 6 patients were anaesthetised without N_2O. These and the 30 patients undergoing routine surgery had no problems. In contrast, the N_2O exposed groups became abnormal with 4 of the 5 patients who received N_2O for only 12 hours having megaloblastic marrows and mean increases in their dU test results from 8.6% to 13.4%. All 6 patients who received N_2O for 24 hours had megaloblastic marrows with a mean dU test increasing from 6.5% to 13.3%. It took 12 hours for

the dU test to become normal after N_2O was stopped. Patients receiving folinic acid had no change in their dU tests indicating that folinic acid prevented the megaloblastic changes.

However, Skacel and others (1982) failed to show this in 3 patients who had 4.5, 7 and 24 hour N_2O anaesthetics. All had large increases in dU test results even if folinic acid was given IV or orally. A fourth patient was given 30 mg folinic acid IV every 5 hours and had no change in his dU test after 24 hours N_2O exposure. Amos and others (1984) used a variety of doses. Eleven patients were ventilated for 24 hours with 50% N_2O and each given from 3 - 60 mg IV folinic acid. All had normal marrows before N_2O administration and only one had a raised dU test (13.3%). Six patients received 3 - 36 mg folinic acid and all had megaloblastic marrows and abnormal dU tests postoperatively (mean 22.1% SEM 3.0%). The other 5 were given 60 mg folinic acid in divided doses, at the beginning and after 12 hours. Four were normal and only 1, a seriously ill patient with a liver abscess, had a megaloblastic marrow and an abnormal dU test. It appears that folinic acid in high doses will prevent marrow changes.

Folinic acid has been used to prevent marrow changes occurring in a seriously ill patient who was repeatedly anaesthetised with N_2O. An 18 year old male had a 105 minute anaesthetic with 70% N_2O after being stabbed. He needed a major transfusion and underwent another laparotomy 7 hours later for which he received N_2O in

oxygen for 2 hours. Folinic acid (30 mg IV) was given at induction and afterwards he received 30 mg IV daily. A marrow sample before induction showed a dU test result of 18.4%, but when repeated 4 hours after the operation was normal at 9.4% Hence in this patient, folinic acid both reversed the abnormality, but also prevented it re-occurring.

Methionine reverses neurological damage due to prolonged N_2O inhalation (Scott and Weir 1981) and it can also correct abnormal dU tests in vitamin B_{12} deficient marrow cells (Sourial and Brown 1983). Theoretically, this compound may prevent haematological depression after N_2O inhalation although it has not yet been evaluated.

Therefore, prolonged exposure to N_2O causes a megaloblastic-like marrow depression. Is this clinically relevant to modern anaesthetic practise? The depression is related to both the duration and concentration of N_2O exposure. Exposure to 50% N_2O for less than 6 hours causes no obvious abnormality and after this, the megablastosis becomes worse with increasing exposure (Amess and others 1978, O'Sullivan and others 1981, Kano and others 1981 Skacel and others 1983). Prolonged intermittent exposure can cause megaloblastosis (Nunn and others 1982) although not all cases show this (Thomson and others (1976), Petrovsky and Yasuni 1965, Fink 1967). Severely ill patients may be more susceptible to N_2O (Amos and others 1982) and folinic acid in high doses can reverse its effects (O'Sullivan and others 1981, Skacel and others 1982, Amos and others 1984). However, the importance of

these findings is difficult to assess. As will be discussed later, changes in marrow function are a late indication of toxicity and it is possible that deleterious changes could be occurring without alterations in the dU test.

Assessment of bone marrow changes from prolonged exposure to N_2O pollution in humans has been made both biochemically and epidemiologically. Biochemical studies have assessed the effects of N_2O upon the bone marrow of both theatre workers and dentists. Salo and others (1984) examined the blood of theatre workers. They studied 8 male anaesthetists and 118 theatre nurses who worked in scavenged theatres. An additional, 10 nurses and doctors who worked in an unscavenged theatre were also examined. All had normal haematological profiles except for 2 anaesthetists who had raised mean corpuscular volumes and one who had 5 segmented neutrophils. However, this study would only detect severe problems because abnormal neutrophil appearance is a late effect of bone marrow depression. Sweeney and others (1985) examined N_2O pollution in dentists using a more sensitive assay. They studied the bone marrow of 21 dentists who used N_2O over a 3-11 week period. Their N_2O exposure was calculated using a personal sampler and an average exposure was 159 to 4000 ppm. Two of the dU tests were abnormal with a third being slightly irregular. The 2 dentists with an abnormal dU tests also had abnormal blood films with bone marrows which appeared megaloblastic on microscopy. All 3 dentists were exposed to high concentrations of N_2O (time averaged

exposure of 1900, 2500, and 1800 ppm) and calculation of total exposure doses showed that they had the 1st, 3rd and 5th greatest exposures. Nunn and others (1982) found no change in plasma methionine concentrations in anaesthetists exposed to N_2O although this is a crude assay of MS inhibition because many factors can influence it.

These biochemical studies suggest that theatre workers are not adversely affected by N_2O pollution. However, 2 of the studies were too insensitive to detect any subtle changes. The 3rd study which examined dentists' bone marrows was positive in 3 individuals. Dentists tend to be exposed to greater amounts of N_2O and it is possible that high concentrations are necessary before toxicity occurs.

There is evidence that N_2O can depress bone marrow other than by direct inhibition of cellular division. It may change the micro-environment of haemopoietic cells. Mice inhaled 50% N_2O for 7 days and then were totally irradiated. Bone marrow cells were injected and the amount that fixed in the animals' bone marrow and spleen were measured. There were no differences in implantation rates in the bone marrow between N_2O exposed and control mice, but a 60% reduction occurred for the spleen (Suzuki and others 1989). N_2O had a greater effect in reducing the spleen stem cells numbers when compared to the bone marrow (Kondo and others 1987). Therefore, does N_2O have effects on the spleen micro-environment in mice resulting in alteration in proliferation and differentiation of stem

cells?

4. Spermatogenesis

Spermatogenesis involves cellular replication and because this requires large amounts of fresh DNA, it my therefore be especially vulnerable to N_2O. Kripke and others (1976) examined 135 young male rats exposed to 20% N_2O either continuously or for 8 hours a day. Tests performed after 1-35 days of exposure showed that testicular weight was decreased after 28 days exposure. Histologically, there was damage to the spermatogenic cells and suppression of spermatogenesis although the supporting cells were completely normal. The smallest exposure which caused this was 2 days both in the intermittent and continuously exposed groups. After 14 days all rats showed some abnormalities, although it was less in the intermittently exposed rats. Recovery took 6 days. Hence N_2O does reversibly decrease spermatogenesis in a dose dependent manner. Because testosterone concentrations remained constant, N_2O appears to act directly on dividing cells.

Rat testis contains MS and this is inhibited by N_2O. After 1 hour exposure to 10 or 50% N_2O, MS activity decreased by 29 and 63%. Full recovery took 24-48 hours in the 10% group and 72 hours in the others. Therefore it is likely from the above study that N_2O has its effects via MS inactivation.

Coate and others (1979) examined young rats using

trace concentrations of N_2O (50 and 500 ppm) and halothane (1 and 10 ppm). They were exposed for 7 hours daily, 5 days a week for up to 52 weeks. Increases in chromosomal damage was seen in both bone marrow and sperm cells. It is interesting that these changes occurred in both groups even though rat MS was not affected by concentrations of less than 450 ppm (Sharer and others 1983). Could there be another factor impairing spermatogenesis?

Other studies have not shown these abnormal effects. Mazze and others (1983) exposed male mice to 0.5%, 5% or 50% N_2O for 4 hours daily, 5 days a week. After 14 weeks there was no change in their testes. Land and others (1981) found no increase in abnormal sperm when mice were exposed to 8% or 80% N_2O for a similar time. Therefore, it appears that N_2O in animals causes a reduction in sperm numbers, but has little effect on sperm morphology.

Are humans affected by N_2O in trace concentrations (or combined with other anaesthetic agents)? Wryobek and others (1981) examined semen samples from 46 anaesthetists who had been exposed to N_2O for at least one year. No abnormalities in sperm concentration or morphology were seen. The operating rooms used scavenging equipment and N_2O concentration varied from 5-300 ppm. Studies on personnel exposed to greater concentrations have yet to be performed.

Overall, it appears that while N_2O causes abnormal spermatogenesis in rats, it has little effect on morphology. Anaesthetists showed no obvious effect, although personnel exposed to higher concentrations have

not been assessed. Individuals undergoing anaesthesia are unlikely to be affected in a meaningful manner because the changes appear to be reversible.

5. Fetal Development

Conception and fetal development require large amounts of DNA and it is possible that N_2O may interfere in three ways. It may decrease conception rate, increase spontaneous abortions or may cause developmental abnormalities. Pregnant women are exposed to N_2O either at work or during a co-incidental anaesthetic. Animal studies have examined the possibility that N_2O can cause increased teratogenicity although interpretation of these results is difficult. Most studies have used small numbers of animals reducing the power of these experiments and extrapolation to other species is impossible because the relative susceptibilities of each animal to N_2O is hard to predict (Cohen 1980). However the experiments do give useful information on any potential problems and their possible causes.

There are many teratogenic mechanisms (mutation, interference with cell division, lack of energy or precursors for development, enzyme inhibition, cell membrane changes). N_2O was shown to be potentially fetotoxic and teratogenic in 1967 when pregnant rats inhaling 50% N_2O had an increase in fetal resorption, mortality and visceral and skeletal abnormalities (Fink

and others 1967). Bussard and others (1974) found similar results when hamsters were exposed to 60% N_2O and 0.6% halothane. This is specifically caused by N_2O rather than a general toxicity of anaesthesia because xenon, a gas with similar properties to N_2O, caused no teratogenicity in similar doses (Lane and others 1980). Further work has been carried out to find when teratogenicity is most likely in pregnancy and how much exposure is needed. Shephard and Fink (1968) exposed pregnant rats to 24 hours continuous 50% N_2O from day 5 to 11 of pregnancy and the incidence of skeletal abnormalities was maximal on day 11, but it increased from days 5-8. Hence, it appears that N_2O has different effects at different times during rat pregnancy.

There is a trace concentration below which no teratogenicity occurs. Pregnant rats were exposed to 100, 1000, or 15,000 ppm N_2O either continuously or for 8 hours a day on days 8-13, 10-13, 12-19, 14-19 or 10-19 of pregnancy. Intermittent exposure had no effect except during days 10-13 when the fetal death rate increased. In the continuous group, 1,000 and 15,000 ppm exposure for 24 hours caused increased fetal death rates with decreased implantations in contrast to 100 ppm which had no effect (Corbett and others 1973). Exposure of rats to 250 or 500 ppm N_2O throughout pregnancy had no effect whilst 1000 ppm caused a difference in the litter size, frequency of fetal resorption and fetal weight (Vieira and others 1980). Hence, in rats, it appears that concentrations greater than 500 ppm are required for teratogenesis. This dose is

similar to the lowest concentration that inhibits rat liver MS activity (450 ppm - Sharer and others 1983). In intermittently exposed rats the threshold was above 1,000 ppm (Vieira and others 1983).

However, not all studies show N_2O to be teratogenic. Pope and others (1978) exposed Sprague Dawley rats for 8 hours daily throughout pregnancy to either a graded concentration of halothane (0.16-0.32%), N_2O (1-80%) or a N_2O:halothane mixture (10% and 0.16%). There was some fetal growth retardation (3-21% decrease in weight), but no increase in fetal loss or skeletal abnormalities. Mazze and others (1982) exposed Swiss ICR mice to 0.5%, 5% or 50% N_2O for 4 hours daily from day 6 to 15 of pregnancy. The 154 female mice studied produced a total of 1,761 fetuses and there was no increase in fetal resorption or skeletal abnormalities.

Why N_2O is not always teratogenic is still unknown. The two negative studies used intermittent exposures and recovery may have occurred during the rest periods although MS activity takes days to recover (Deacon and others 1978). N_2O was given throughout pregnancy and there may have been sufficient time for alternative pathways of folate metabolism to be induced. Rats have a betaine pathway which converts homocysteine to methionine and this requies several days days before it is induced (Lumb and others 1983). Davidson and others (1988) grew thymidine kinase negative mutant mouse leukemic lymphoma cells without methionine and then exposed them to 76% N_2O or N2 for 30 minutes. Cell growth was identical although there

was no MS activity in the N_2O cells. Because these cells are incapable of making deoxythymidine from thymidine it suggests that alternative methods of methylating deoxyuridine MS exist. Rat exposure to N_2O in early pregnancy may allow induction of this pathway and no teratogenicity will occur.

The mechanism of N_2O teratogenicity is still unknown. Maternal and fetal MS was inhibited in a dose dependent manner although it was slower in the fetal enzyme probably because of the time taken for N_2O equilibration between maternal and fetal blood (Baden and others 1984). In vitro N_2O inactivation of fetal MS was similar to that of maternal MS (Baden and others 1987). Is the teratogenicity a consequence of abnormal folate and DNA metabolism? N_2O does decrease cellular proliferation in mice fetuses exposed briefly to N_2O (Rodier and others 1986). However there is evidence that other mechanisms may be responsible. If isoflurane (0.35%) and N_2O (50%) are given to rats the effects are substantially reduced (Fujinaga and others 1987a), but fentanyl alone had no effect (Mazze and others 1987). If teratogenesis is mediated via folate inhibition, folinic acid should protect the animal although there was no change (Keeling and others 1986, Fujinaga and others 1987B). N_2O causes a high adrenergic tone and increases plasma noradrenaline concentrations (Smith and others 1970) and this may reduce uterine blood flow and compromise the fetus. Because both isoflurane and halothane prevent a high adrenergic tone and cause arterial dilation, it is possible that they can reduce the

vasoconstrictor effects of N_2O. However, in a culture system 100 10 day old rat embryos exposed to 75% N_2O had a reduced DNA content compared to controls and 7 rats developed abnormally (Fujinaga 1988). Exposure of rat fetuses to 75% N_2O on day 9 in the same system increased abnormalities (Baden and Fujinaga 1991). This whole embryo culture system avoids maternal influences and allows the direct effects of N_2O on the fetus to be assessed. Are the effects of N_2O multifactorial?

There have been several epidemiological studies which have examined the effects of anaesthesia and surgery in pregnant women because it is possible that anaesthesia during early pregnancy may cause an increase in spontaneous abortions. There was an incidence of 8.5% and 9.3% respectively in fetal death and congenital abnormalities in 147 women who had had surgery in the first trimester (controls were 2.0 and 6.0% respectively)(Schnider 1965). A postal review of dentists and dental assistants showed that of 187 pregnant women who had been operated on during the first trimester and 100 in the second, 9.6 and 2.6% respectively had aborted. Control values for these 2 groups were 5.1 and 1.4% (Brodsky and others 1980). A review of 175 pregnancies during which an anaesthetic (20 - 30 minutes) was given for the insertion of a Shirodkar suture (mostly between 14-16 weeks) was unable to show any teratogenicity (Aldridge and Tunstall 1986). Slater (1970) described a patient who gave birth to a normal boy, even though she received 17 anaesthetics during her pregnancy. A

retrospective study reviewed 53 infants who had neural tube defects and 97 with other major congenital abnormalities. Only 1 mother had received an anaesthetic. The anaesthetic histories of 471 mothers showed that 72 of then had received a total of 76 anaesthetics, 70 before their last menstrual period and 6 during pregnancy. No abnormalities were found although 2 miscarried (Konieczko and others 1987).

Other studies have examined larger numbers of women and found a similar high rate of miscarriage after anaesthesia in the first trimester. Duncan and others (1986) assessed more than 1,000,000 people who lived in Manitoba from 1971 to 1978 and found 2,565 women who had undergone surgery during pregnancy. Each woman was matched to a control. In the surgical group, there was a 1.68% incidence of congenital anomalies and a 7.1% abortion rate compared to controls of 1.52 and 6.5% respectively. The type and timing of the anaesthetic had no effect on congenital anomalies although general anaesthesia increased the number of abortions significantly (risk ratio=1.58). Whilst the evidence is still not definitive, it does appear that surgery can increase the incidence of abortions. Is this due to N_2O or to other factors (eg. volatile anaesthetics).

Does N_2O have an effect on fertilisation? Ninety-eight patients received either N_2O and isoflurane or isoflurane alone for laparoscopic retrieval of oocytes and both groups had the same successful fertilisation rates suggesting that N_2O has little effect. This is expected

because oocyte retrieval is a relatively quick procedure and N_2O exposure is only brief. However, prolonged N_2O exposures may interfere with fertilisation and the implantation rate? Should N_2O be used in women attempting to have a family?

Does N_2O have an effect on fetuses when used for Caesarean section late in pregnancy? Fetal MS is inactivated in N_2O exposed rats (Baden and others 1984, 1986). However, a similar experiment would be impossible to do in human fetuses. Landon and Toothill (1986) examined MS activity in placentae collected either after Caesarean section (with a N_2O exposure of 13 to 22 minutes) or after normal vaginal delivery and found no difference in MS activity.

Overall, the evidence indicates that short N_2O anaesthetics for pregnant women are probably safe after the first trimester although they may cause an increase in first trimester abortions. The embryo culture system indicates that N_2O directly affects the fetus. It may be advisable to avoid N_2O as an anaesthetic for women in the 1st trimester of pregnancy. Whether N_2O should be used in women attempting to become pregnant has not yet been established.

Fetotoxicity and teratogenicity may occur in exposed female workers (Askrog and Harvald 1970, Cohen and others 1980, Erikson and others 1979, 1985 Tomlin 1979, ASA 1974, Corbett and others 1974, Knill-Jones and others 1972). Initial studies showed there was an increase in spontaneous abortions in these women although the study

sizes were small and controls were inadequate. Buring and others (1985) pooled the results of 6 studies to calculate the RR for women. For spontaneous abortion the RR was 1.30 (95% CI 1.2-1.4) and for congenital abnormalities it was 1.20 (95% CI 1.0-1.4). Nurses were less affected than doctors. It is possible that this small increase in RR may have been a result of bias in the studies. Cohen and others (1980) assessed the effect of N_2O upon pregnant women and showed a dose dependent increase in spontaneous abortions of up to 105% above control values for the chairside assistants. Also, there was a small increase (50%) in congenital abnormalities among children of the same chairside assistants. However, this was not dose dependent.

In Sweden a Medical Birth Registry, started in 1973, holds information on pre-natal care, delivery and post-natal examinations for 99% of pregnancies. Similarly, a registry for nurses for abortions (spontaneous and induced) exist. Using these registries, Ericson and Källen (1979) studied 494 pregnancies from 1973-1975 in women who had worked for at least 50% of their pregnancy in theatres. The data was accurate and verifiable because there were no responder problems. No abnormalities were seen except for a shorter gestation period. Similar work on the same registries over a longer period (1973-1978) involved 1323 theatre nurse. Controls were 1382 medical ward nurses and results were similar (Ericson and Källen 1985). The Nurse Registry allowed a case-control within-group study. Twenty five theatre nurses whose children had

died perinatally or had severe congenital malformations were chosen. Controls were 50 nurses from the same group who had had a normal childbirth. Questionnaires sent to these 75 women (74 replies) showed no difference.

Hence, it appears from these epidemiological studies that the toxic effect of exposure to N_2O pollution is not as significant as was once thought with the only possible abnormality being a slight increase in spontaneous abortions in pregnant females.

6.Infection

It is possible that N_2O may affect the immune system. Normal human lymphocytes exposed to N_2O for a short period had a reduced methylcobalamin synthesis, a reaction that is vitamin B_{12} dependent (Linnel and others 1978). Prolonged exposure to N_2O causes a peripheral leukopenia due to marrow depression from inhibition of DNA synthesis and several deaths have resulted from this (Lassen 1956). Is it possible that N_2O could have more subtle effects? Leukocytes (neutrophils, monocytes and lymphocytes) fight infection by either phagocytosing and killing bacteria or by producing antibodies. For the proper fuctioning of the immune system sufficient leukocytes must be made and then mobilised from the body's stores. They must be able to undergo chemotaxis to the bacteria, kill and then phagocytose them.

Depression of chemotaxis leads to an increase in susceptibility to infection. Nunn and others (1982)

investigated the motility of human neutrophils in vitro. Neutrophils were exposed to 80% N_2O in oxygen and their chemotactic response towards casein found. Compared to controls, this decreased by 22% and this may explain why a leukopenia develops after 24 hour N_2O exposure even though there are sufficient body stores to last 72 hours (Skacel and others 1983). Fresh neutrophils are unable to be mobilised into the blood and because their life span is brief, a deficiency will soon develop. However, neutrophil exposure to volatile anaesthetics for 24 hours caused a similar depression. One MAC isoflurane, enflurane, halothane and methoxyflurane reduced motility by 8, 32, 50, and 58% respectively compared to a reduction of 53% with 0.76 MAC N_2O. (Moudgil and others 1984). Is the depression a general effect of anaesthesia rather than a specific effect of N_2O on MS?

In vivo experiments have given a different result. Seven volunteers had blood sampled before and 20 minutes after inhaling 60% nitrogen and again 20 minutes after inhaling 60% N_2O. This was inhaled for 60 minutes and 20 minutes later a final sample was taken. All the samples were tested for polymorphonuclear (PMN) chemotaxis, PMN random motion and white blood cell and PMN concentrations. N_2O increased the PMN chemotactic index, but had no effect on any of the others (Hill and others 1978). Alpha adrenergic agonists enhance chemotaxis whilst beta agonists reduce it (Hill and others 1976). Exposure to N_2O increases the concentration of plasma noradrenaline by 40% (Eisele and Smith 1972) and this may be responsible

for the stimulating effects of N_2O *in vivo* as compared to its inhibitory effects *in vitro*. Hence, even if N_2O *in vitro* causes a decrease in motility and chemotaxis, then is it clinically relevant?

Phagocytosis appears unaffected. *In vitro* exposure of human leukocytes to either 0.5%, 2.5% or 80% N_2O for 30 minutes didn't affect the phagocytosis of latex particles (Cullen 1974). Welch and Zaccaria (1982) looked at the effects of N_2O on the oxidative activity of human neutrophils. Neutrophils can generate reactive oxygen species (superoxides and peroxides) which kill bacteria. Eighty percent N_2O had no effect on their generation. Nitroblue tetrazolium was reduced in a normal manner by leukocytes exposed to 80% N_2O for 30 minutes indicating normal oxidative activity (Cullen 1974).

N_2O can cause an *in vitro* dose dependent reduction in killer cell cytotoxicity although halothane or enflurane have greater effects. Withdrawal of N_2O reverses this effect (Woods and Griffith 1986). In conclusion, even though the effects of N_2O are not consistent, there appears to be a slight effect on the immune system. There is a reduction in cell production and a decrease in blood leukocyte concentrations, a decrease in the ability to mobilise and undergo chemotaxis although this may not occur *in vivo*. Phagocytosis and the ability to kill bacteria is unaffected. Some of these effects may be due to a direct effect of anaesthesia and surgery rather than a specific N_2O effect. The incidence of post-operative wound infections increase with increasing surgical

duration and therefore increasing duration of exposure to N_2O (Cuse and Foord 1973) although there are many other explanations for this. A blinded study which would examine the effect that N_2O has on wound infection is the obvious study to be undertaken, although this is a large undertaking. The clinical consequences of N_2O exposure on the immune system have yet to be established.

7. Wound Healing

A further possibilty is that N_2O may interfere with wound healing because fibroblasts must replicate and produce new proteins for adequate healing. Shah and others (1978) made a 3 cm linear incision in 90 rats; 30 of which were exposed to air, 30 to 20% continuous N_2O and 30 to 20% N_2O 8 hours each day. Ten rats were killed on days 3, 7 or 10. Eighty-seven of the 90 rats had normal wound healing, both grossly and histologically and N_2O had no effect. Algie and others (1985) exposed 24 female rats who had received a 4 cm incision in the paravertebral area to either air or continuous 3% N_2O. Fifteen to 18 days later they were killed and the force required to break the wound was found to be the same in both groups. A second batch of 40 rats were exposed to either Entonox or 50% nitrogen in oxygen. On the 8th day they were killed. No statistical difference was seen in the force required to break the scars between the 2 groups. A similar result was

seen by Parbrook (1967).

Hence, it seems unlikely that N_2O impairs wound healing significantly, although there is a possibility that it may cause minor problems which may increase the effects of other complications (poor nutrition, obesity, poor surgery, etc). A full study of this, similar to that for infections is a large undertaking.

8. Neuropathy

Chronic N_2O exposure can cause a neuropathy in humans. This is an effect unique to N_2O, although possibly not related to MS inactivation. The condition was first seen in 1959 when 2 patients, both with chronic myeloid leukemia, were treated by inhalation of N_2O. Both became euphoric and then depressed. Eventually they became very tired and lapsed into a coma (Lassen and Kristensen 1959).

In 1978, Layzer and others described 3 patients who had abused N_2O whilst Sahenk and others (1978) described a student who had inhaled N_2O from a whipped cream dispenser. All had similar neurological disorders and nerve biopsies showed a peripheral neuropathy. Layzer (1978) described 15 men (aged 22-50 years), 14 of who were dentists and the other a dental technician. All had been exposed to N_2O for between 3 months to 12 years, 13 had self administered it and the cause in the other 2 was faulty equipment. The commonest presenting symptom was a numbness and/or tingling in the hands and feet which

ascended up the limbs. Lhermitte's sign was positive in 12. Other common symptoms included impotence (in 7), abnormal bowel movements (in 4) and depression or memory loss (in 7). Ten had retired from work. Later symptoms suggested the development of a distal sensiro-motor polyneuropathy involving the long motor/sensory tracts of the spinal cord. The EMG showed a denervation pattern in 6 cases and a reduced motor nerve conduction in 10. The CSF, myelography and vitamin B_{12} concentrations were normal. There was an immediate improvement on stopping N_2O inhalation in one patient whilst the others took weeks although none fully recovered.

The condition appeared to be caused by N_2O because no other abnormality or toxic factor was present and vitamin B_{12} concentrations were normal. The neurological picture was unusual and distinctive and it improved on withdrawing N_2O. The syndrome was similar to that in patients with pernicious anaemia, subacute combined degeneration of the cord (SACD).

Two factors have hampered study into this neuropathy. Human work is difficult because the syndrome is rare and only a few suitable animal models exist (e.g. rats when exposed to N_2O do not develop any neuropathy (Dyck and others 1980)). Two animal models that have been used are the fruit bat (Van der Westhuysen and others 1980) and the primate (Dinn and others 1978). Fruit bats develop a neuropathy after only a few weeks exposure whilst primates take up to 10 or more weeks. Although both animals are expensive to buy and difficult to look after they have

been used as models for SACD.

N_2O inactivates B_{12} and because this vitamin is found in only 2 enzymes, it is probable that the neuropathy is due to inactivation of either enzyme. MS is rapidly inactivated by a direct effect whilst MMCoA mutase is inhibited indirectly and this takes longer (Kondo and others 1981). Accumulation of both methyl-malonic acid and homocysteine in the urine of patients with pernicious anaemia is seen indicating that both enzymes are inhibited in this condition (Lindenbraum and others 1988). Whatever the reason for the neuropathy, it must explain why these patients often have no haematological abnormalities (Lindenbaum and others 1988).

1. Inactivation of methyl malonyl CoA mutase (Frenkel and others 1973a 1973b). Folic acid supplementation in vitamin B_{12} deficienct patients worsens the condition (Will and others 1959). MMCoA mutase catalyses the conversion of the branched chain fatty acid, methyl-malonic acid to succinic acid and is required for catabolising proprionic acid, a 3 carbon fatty acid.

Proprionyl CoA + CO2 ----> Methylmalonyl CoA

When this enzyme is inhibited there is an accumulation of both odd chain and branched fatty acids which may then be incorporated into the myelin around axons and cause them to function abnormally. Frenkel

(1973) found both C15 and C17 fatty acids in the sural nerves of patients with pernicious anaemia. Because both inactivation of MMCoA mutase and incorporation of abnormal fatty acids into the myelin is slow it would explain why the neuropathy takes a long time to develop.

However, not all children with a hereditary deficiency of MMCoA mutase have neurological abnormalities similar to those of SACD (Morrow and others 1969). Additionally, Scott and others (1981) demonstrated the protective effects of methionine on N_2O inactivated vitamin B_{12} deficient monkeys. MMCoA mutase is not involved in methionine metabolism and so it is difficult to explain why methionine protects the animals by this theory.

2) Inactivation of methionine synthase (Jacobsen and others 1973). This will decrease the formation of methionine, a vital precursor of S-adenosyl methionine (SAM) a compound required for methylation reactions. If methylation is reduced, it may cause a neuropathy. Cycloleucine inhibits transmethylation reactions of SAM and if fed to rats causes a demyelination syndrome similar to vitamin B_{12} deficiency (Small and others 1980).

Further work was performed on monkeys (Scott and others 1981). Two groups were exposed to 15% N_2O continuously for 10-12 weeks with the diet of one group supplemented by 2 g methionine daily. Throughout treatment the supplemented monkeys remained normal. However, in the non-supplemented group, all the monkeys became ataxic

after 10 weeks and their condition then worsened considerably over the next 3 weeks. At autopsy the classical changes of SACD in the spinal cord and the peripheral nerves were seen. Hence, it appears that methionine prevents these degenerative changes from occurring supporting the theory that it is the inactivation of methionine synthase by N_2O that causes the neuropathy.

SAM concentrations in the brains of rats exposed to 70% N_2O for 24 hours were significantly reduced (Vina and others 1986). However, this was not seen in fruit bats (Van der Westhuyzen and others 1983) and the reasons for this remain unknown.

An interesting theory was proposed by Weir and others (1988). They exposed pigs to 15% N_2O for 3, 5 and 9 weeks and half of them received extra methionine. Those exposed to N_2O without methionine developed ataxia and severe spinal damage was seen at pathology. Brain and liver MS activity was reduced. Their spinal cords showed an increase in SAH concentrations, but SAM concentrations remained normal (SAM:SAH ratio inverted). The supplemented group also had a similar increase in SAH concentrations, but SAM concentrations increased as well (SAM:SAH ratio normal). From these results, it appears that SAM concentrations are not responsible for the neuropathy. This change in methylation ratio (from 15 to 0.8) can inhibit the transmethylation enzymes (Schatz 1977) and this may lead to a failure of methylation. Interestingly, the ratio change in rats is not as dramatic (from 2.7 to

1.4) which may explain why rats do not develop the neuropathy.

Scott and Weir (1981) proposed that it was the methyl folate trap that was responsible for the neuropathy. They suggested that the trap is a normal physiological process whereby cells are able to overcome periods of methionine deficiency which occur during episodes of starvation. It maintains SAM concentrations to ensure vital transmethylation reactions can occur. Methionine concentration is maintained by 2 processes. Firstly, when it is low, so is SAM concentration as well. This results in an increase in 5-methylTHF concentrations because no SAM inhibition of the reduction of 5,10 methylene-THF occurs. This prevents folate from entering the purine/pyrimidine cycle because all folate is in the 5-methyl form. DNA formation and therefore cell division is inhibited. This will conserve methionine. Secondly, for folate to be retained by a cell, the transport form, 5-methylTHF, a monoglutamate, should be converted to a polyglutamate. Folate must be in its unmethylated form before polyglutamation can occur and if folate remains as 5-methylTHF, it will not be retained by the cells and a relative lack of cellular folate will occur. This will decrease cell turnover.

In this type of neuropathy, the theory suggests that the cell interprets vitamin B_{12} inactivation incorrectly. It sees it as methionine shortage and responds inappropriately by trapping all folate as 5 methyl-THF. This will aggravate the decreased supply of methionine and

SAM concentrations will decrease.

As yet, its pathogenesis is still unknown. Whatever the cause, it is rare and prolonged exposure to high concentrations of N_2O are needed. In humans, it only occurs in either self-administered cases or in people who are exposed to high concentrations by accidental malfunctioning of anaesthetic machines.

Assessment of the effects of chronic exposure to trace concentrations of N_2O was made in dentists. Nineteen were examined and they were placed in either a low (no exposure or less than 5 litres.hour^{-1}.week^{-1}) or high exposure group (greater than 5). Neurologically, there was no difference between these groups. However, only a few subjects were studied and because the effect occurs rarely, it could have been missed.

Brodsky and others (1981) examined approximately 30,000 subjects half of whom used inhalational anaesthesia and half who didn't (Cohen and others 1980). Neurological complaints were assessed including symptoms of tingling, numbness and muscle weakness. Exposure to N_2O was classified as none, light (less than 2,999 hours/10 years) and heavy (greater or equal to 3,000 hours/10 years). There was an increase in neurological complaints in both male dentists and female chairside assistants. The overall incidence of complaints was small (less than 2%). Even with the limitations of this study, it appears that occupational exposure to N_2O may be detrimental to health.

Summary

N_2O has the potential to cause multiple effects in humans when exposed either acutely or chronically. However, the true clinical relevance of many of these is still unknown.

CHAPTER FOUR

ASSESSMENT OF THE TOXICITY OF NITROUS OXIDE

Rationale for the study

Assessment of the Toxicity of Nitrous Oxide

How can the biochemical and clinical toxic effects which N_2O may have on humans be measured? The use of experimental animal models has allowed us to theorise how these effects may occur, but we are unable to extrapolate the results to man. There is a species difference in the rate that methionine synthase (MS) is inhibited by N_2O with human MS being relatively resistant. In addition, animals develop different clinical problems after exposure when compared to man. Only a few species have similar neurological symptoms to man after chronic N_2O exposure and we are the only species to suffer from megaloblastic anaemia. Animal studies will give little indication of the possible consequences of N_2O exposure.

Therefore, to assess the clinical consequences of exposure, man must be studied and 2 different techniques, epidemiological and biochemical, have been used. The many epidemiological studies examining N_2O exposure in both patients and staff have not been able to provide definitive answers. Biochemical assays are of two types and both require tissue samples. One techniques measures human MS activity and the other assesses bone marrow for megablastosis. Unfortunately, because MS is not found in blood, invasive tissue samples are needed with the liver and bone marrow being the favourite organs. Because invasive sampling has potentially devastating consequences, only a small number of patients have been

examined. In addition, the large variations that exist between human MS activity has made calculation of the average inactivation $t_{\frac{1}{2}}$ difficult. For example, whilst mean human MS $t_{\frac{1}{2}}$ of inactivation by 70% N_2O is 46 minutes, its 95% confidence limits are 30 - 99 minutes (Royston and others 1988). Another problem in assessing the clinical importance of MS inactivation is how much MS must be inhibited before any relevant clinical signs or symptoms appear? It is possible that no detrimental effects will occur even if most of the MS in the body is inhibited.

The extent of bone marrow megablastosis can be assessed both by microscopy or by using the dU test. Again an invasive marrow aspirate from either the sternum or the iliac crest is needed and consequently, only a few patients have been tested. A positive test indicates that an effect detrimental to the patient has occurred. However, other diseases can cause megaloblastosis and therefore this test's specificity is poor. It is unable to detect whether N_2O has toxic effects prior to megablastosis occurring and so suffers from a reduced sensitivity. Other problems occur with microscopy because of its subjective nature including the missing of minor changes and making comparison between samples difficult. The dU test is more objective and gives a quantitative assessment of marrow depression. However, it is a difficult assay to set up and each laboratory has its own normal range making comparisons between studies difficult. Normal values are often arbitrary with some laboratories having a 10% dU limit. If the result is 9.9%, does this

indicate pre-clinical toxicity or is the marrow normal? Toxicity is not all or non, but a spectrum.

Because the main biochemical methods used to assess N_2O toxicity have only been able to examine small numbers of patients, their results must be treated with caution. Other biochemical assays have been used which are less invasive although are also equally unsatisfactory.

Measurement of plasma methionine and S-adenosyl methionine concentrations have already been discussed. Short term anaesthesia (25 - 217 minutes) caused no change to either although pre-operative starvation did decrease methionine concentration (Nunn and others 1986). In contrast, prolonged exposure (8 - 24 hours) reduced methionine concentration to 15-25% of control values (Skacel and others 1983). Anaesthetists exposed to N_2O had normal concentrations (Nunn and others 1982). However, other factors are important in methionine metabolism including the individual's diet and the stress of surgery and it is difficult to know the significance of this reduction.

When MS is inhibited, plasma homocysteine concentration may increase because it cannot be converted to methionine. Bevan and others (1982) examined the urine of 41 patients undergoing N_2O anaesthesia (1 to 18 hour duration) by collecting a sample voided after surgery. A simple nitroprusside colorimetric test was used to measure homocysteine. This reacts with disulphides formed from the reaction of homocysteine and cyanide to give a magenta colour with a positive test occurring if there is more

than 0.12 $\mu M.ml^{-1}$ homocysteine present. No colour change occurred in any of the samples indicating no homocystinuria. However, this test is relatively insensitive and readily gives a false negative result. In addition, homocysteine is metabolised in a variety of other ways. Recently, a radioactive assay system for accurately measuring plasma homocysteine concentration has been descibed and this may allow the effects of long term N_2O exposure on homocystinuria to be studied (Cho and Hall 1988).

Rask and others (1983) studied the effects of N_2O upon methylmalonylCoA (MMCoA) mutase by looking at the urinary excretion of methyl malonic acid in 5 patients who had received 50% N_2O. Urine was collected for three 24 hours periods, before, during and after the operation. Control values were found using haematology nurses. A dU test performed after N_2O ventilation was abnormal. Urinary methyl malonic acid excretion increased 3 fold during N_2O exposure becoming normal after N_2O was stopped. However, the control and exposed subjects were not comparable and the increases in excretion could have been due to the stress of the operation (open heart surgery) or to the anaesthetic. MMCoA mutase was shown by Kondo and others (1983) to be inhibited by N_2O although only after prolonged exposure (16 days). Therefore it is unlikely that N_2O affects this enzyme during a relatively short anaesthetic.

Overall, the assays used to measure possible N_2O toxicity are unsatisfactory being either clinically

irrelevant, having an inadequate specificity or sensitivity or requiring invasive sampling. As yet no completely suitable assay system has been developed and this is the rational for these series of experiments. It is an attempt to find a repeatable, quantifiable and meaningful method of assaying N_2O toxicity in a variety of exposed groups.

Because folate metabolism is the link between MS inhibition and clinical toxicity, its concentration may be used as an indicator of N_2O toxicity. This should be more sensitive at diagnosing clinical toxicity than bone marrow tests because an abnormality in folate metabolism will occur before the marrow is depressed. It may also be of more clinical relevance than MS activity because folate affects many different metabolic processes and any abnormalities may indicate a derangement of bodily function.

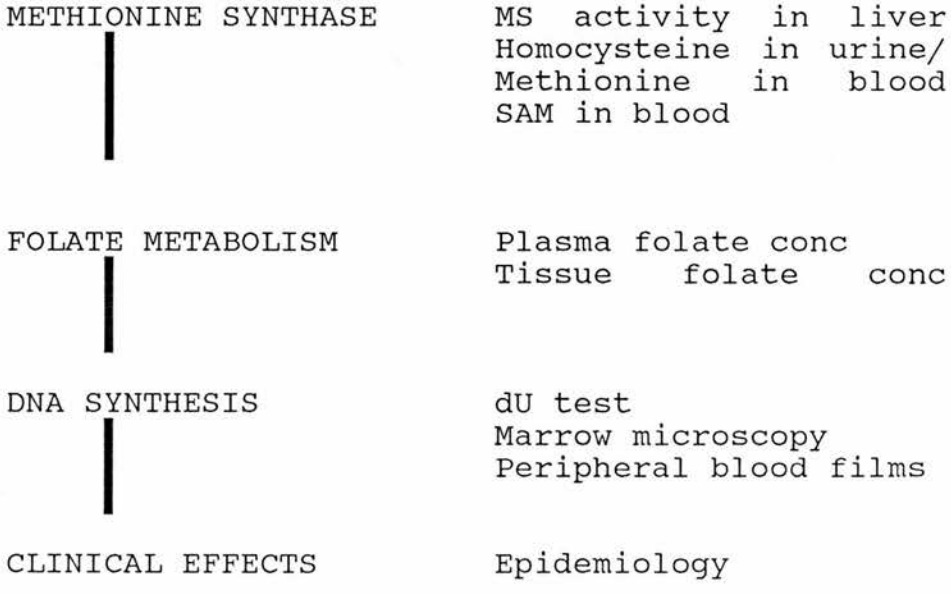


Figure 4. methods of investigating N_2O toxicity.

How can folate metabolism be measured? Animal work has shown the effects that N_2O has on the concentration of each folate co-enzyme. Total plasma folate concentration measured in man progressively increases with increasing N_2O exposure (Skacel and others 1983). However, cellular folate status is more important than plasma concentrations and because tissue samples are too invasive for frequent use another method of assessing cellular folate metabolism is required.

Various tests have been used. Therapeutic trials with oral folate were found to be too time consuming and it was difficult to calculate an absolute folate deficiency (Marshall and Jandl 1960). The plasma clearance and urinary excretion of tritiated folic acid (^{3}H-Folic acid) allows folate deficiency to be diagnosed within 3 hours although the test is relatively crude and too insensitive to detect minor abnormalities (Sheehy and others 1962). Other tests used include measuring the concentration of urinary amino-imidazolecarboximine. Folate is essential for converting this to AICAR and when there is a folate abnormality it accumulates and is excreted in the urine (Johns and Bertino 1965).

Another method of assessing folate metabolism is the formiminoglutamic acid urine excretion test. This test assesses the ability of the body to metabolise histidine, a process which requires an intact folate metabolic pathway. This test is able to indicate the functional state of cellular folate metabolism with an increase in

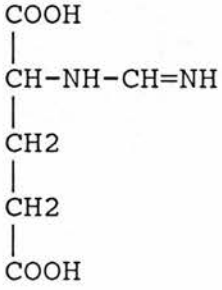
urinary formiminoglutamic acid excretion indicating an abnormality in folate metabolism.

The Formiminoglutamic Acid Urine Excretion Test

In the late 1950's there was much research into developing a suitable assay system to assess folic acid deficiency. Measuring plasma folic acid blood concentrations was time consuming and gave confusing results because each laboratory used its own control values (Stokstad 1943, Schweigert and others 1947, Usdin and others 1956, Chanarin 1958). Another method was to measure gastrointestinal absorption of folic acid (Chanarin and others 1958). However, it became apparent that the only reliable diagnostic test was a clinical trial whereby the patient was given folic acid and his clinical progress charted. This was a laborious and time consuming process.

However previous work suggested there might be another possible assay system to measure folic acid deficiency *in vivo*. Bakerman and others (1951) noticed that rats deficient in folic acid excreted large amounts of a glutamic acid precursor in the urine which if heated allowed a bacteria, Lactobacillus arabinosus, to grow. Further work showed that this urinary compound was formiminoglutamic acid (Silverman and others 1952, Tabor and others 1953, Borek and others 1953, Seegmiller and others 1954, Broquist and Luhby 1959).

Formiminoglutamic acid (FIGlu) is a small, water soluble molecule (mol. weight 183). Structurally, it consists of a glutamic acid moiety with a formimino group (-CH=NH) attached to its 4th carbon.



Formiminoglutamic acid (FIGlu)

FIGlu excretion was proportional to the severity of folic acid deficiency. Tabor and others (1953) used ^{15}N-histidine to show that 55% of body histidine was converted to glutamic acid with the nitrogen on the amino group of FIGlu coming from the imidazole ring of histidine. This catabolism occurred in both bacteria and liver extracts. An intermediate in the pathway was urocanic acid and further work discovered the histidine catabolic pathway (Slavik and others 1954, Tabor and Mehler 1954, Sagers and others 1956, Tabor and Rabonowitz 1956, Miller and Waelsch 1956, 1957).

histidinase Urocanase imidazolone-proprionate hydrolase

HISTIDINE ----> UROCANIC ACID ----> 4-IMIDAZOLE,5-PROPRIONIC ACID ----> FORMIMINOGLUTAMIC ACID

It was noticed that THF was necessary to convert FIGlu into glutamic acid by accepting its formimino group in both mammals and bacteria. The enzyme FIGlu transferase

catalyses this reaction (Tabor and others 1956).

```
FIGlu                          5-formiminoTHF
  +          ------->              +
THF                            Glutamic acid
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Histidine, an amino acid, is essential for infants (Snyderman and others 1963), but not adults (Rose and others 1951). It is catabolised in a variety of ways (Stifel and Herman 1971) although the most important pathway is to FIGlu. Others include;

1) Decarboxylation to histamine, then oxidation to imidazoleacetic acid.

2)Transamination to imidazolepyruvic acid, which is reduced to imidazoleacetic acid. This is the second major pathway of degradation.

3) Methylation to 1 or 3-methyl histidine.

4) Condensation of histidine or methyl histidine with B-alanine to form carnosine or anserine respectively.

It was considered that measurement of urinary FIGlu excretion would be able to quantify the folic acid metabolic state in humans because shortage of THF causes a buildup of FIGlu. Being water soluble, this compound is excreted in the urine. Initial evidence supported this view. Leukaemic children prescribed a folic acid antagonist had an increased urinary excretion of glutamate activity which decreased when the antagonist was stopped (Broquist 1956). Luhby (1957) showed a similar increase in a pregnant lady with macrocytic anaemia that

decreased with folic acid therapy. Further work confirmed these observations (Luhby and others 1958) and that the increase in glutamate activity was caused by an increase in FIGlu concentrations (Broquist and Luhby 1959).

In early studies of folic acid deficient people it was common to see very low urinary FIGlu excretion even if a haematological response had occurred after folic acid supplementation. This was possibly because of a shortage of histidine in the body. To overcome this, metabolic loading with oral histidine was tried. Patients swallowed histidine monochloride in large doses. Because the average daily histidine intake is 0.5-2.0 gm (Orr and Watt 1957), greater amounts will allow any dietary variations to be ignored. With 15 gm oral histidine given daily for 48-72 hours, normal patients excreted less than 0.15 $\mu mol.ml^{-1}$ FIGlu compared to those with a folic acid deficiency. These patients had a 3 to 1000 fold increase in excretion. The urine was collected over 24 hours (Luhby and others 1958, 1959b).

Hence, it appeared that histidine loading made the test more sensitive. Various regimes were tried using different amounts given either once or in divided doses. With no loading folic acid deficiency can only be diagnosed if urinary FIGlu concentration is greater than 0.05 $\mu mol.ml^{-1}$ (Luhby 1960). Doses of 15 gm daily in 3 divided doses 4 hours apart was the most specific method of assessment although it was accompanied by a high incidence of nausea and vomiting. (Luhby and Cooperman 1964). Doses greater than 25 gm in normal subjects for

several days resulted in abnormally high FIGlu excretion presumably due to an overloading of the catabolic pathway (Luhby and Cooperman 1959). Doses of 10 to 20 gm allowed folic acid deficiency to be diagnosed when there was little or no megaloblastosis present in the marrow (Luhby and others 1958, Luhby and others 1959).

After histidine ingestion any FIGlu produced is mostly excreted within 8 hours. It takes 2 hours before it appears in the urine with a peak excretion at 6 hours. Only small amounts are excreted after this (Kohn and others 1961, Chanarin and others 1962, Knowles 1962, Knowles and others 1961). Therefore, the most efficient collection period is 8 hours after loading. Histidine monochloride when dissolved in water has an unpleasant taste, similar to that of stagnant water. It is also poorly soluble with its crystals dissolving slowly (Chanarin and others 1963). The best method to give it is in warm water or orange juice.

Chanarin and others (1963) and Davis and Kelly (1963) showed that urocanic acid was often excreted in large quantities in the urine in addition to FIGlu. However there appears to be little correlation between folate deficiency and urocanic acid excretion (Knowles 1961). Perhaps the raised urocanic acid excretion found by these investigators was due to a buildup in the metabolic pathway of histidine. Alternately there could be a decrease in liver urocanase activity due to an inhibitory feedback mechanism by FIGlu (Baldridge 1958). Thirdly, it could be an artifact of the enzymatic assay used to

measure FIGlu because histidinase is often present in the enzyme preparation and this enzyme could metabolise any histidine present in the urine.

The clinical application of this test was not used immediately because measuring urinary FIGlu is difficult. There are now 4 main measurement techniques, all of varying complexity and sensitivity. They are microbiological, electrophoretic, enzymatical and chemical.

1)Microbiological Assay

When Lactobacillus arabinosus is grown in a synthetic broth containing all required growth factors except glutamic acid, any growth occurring is related to the amount of glutamate (glutamic acid or glutamine) added (Henderson and others 1948). FIGlu causes no growth unless heated because it then decomposes to glutamic acid (Silverman and others 1952). Hence the urinary concentration of FIGlu can be found by measuring growth when heated urine is added. Heated urine is added to the broth and L.arabinosus growth is measured using the turbidity of the solution. Normally, urine contains 400% more glutamine than glutamic acid and when this is heated, it degrades to pyrollidone carboxylic acid. This does not support growth and hence the ratio of glutamate activity of heated to unheated urine (H/U) is usually about 0.3. However, if FIGlu is present this ratio increases and values greater than 0.9 are considered significant (Broquist and others 1959). Controls have been found

using this assay on normal patients and on two folic acid deficient patients, a child with acute leukaemia treated with anti-folate drug and a pregnant women with macrocytic anaemia. Both had an increase in the H\U ratio.

To perform the assay, urine is sterilised by filtration and two aliquot taken, one being heated. They are added to samples of the broth and growth assessed. Standards can be found using known amounts of glutamic acid. The test is reasonably specific and reproducible, is moderately easy to perform, although it is an indirect assay. Its sensitivity is moderate being able to measure FIGlu concentrations of 10 $\mu g.ml^{-1}$. Disadvantages include the time taken to complete the assay (96 hours) and colour in the urine can influence the test, although activated charcoal will remove most of the colour compounds. Additionally, with a low urine glutamine content, high H/U ratios can be found even when FIGlu concentrations are low.

A modification of this test by Davis and Onesti (1960) gives a greater sensitivity but it still requires 6 days to complete. They allowed L.arabinosus to grow for a day with the addition of unheated urine to extract all the glutamate activity. The bacteria were removed by centrifugation, the sample reheated and re-assayed. The sensitivity is about 5-10 $\mu g.ml^{-1}$.

2) Paper Chromatography

FIGlu moves faster than glutamic acid or glutamine in a descending solvent front paper chromatography. Tabor and

others (1954) took 18 hours to estimate FIGlu content with chromatography. FIGlu has no free amide groups but if exposed to ammonia vapour, the formimino group is displaced and ninhydrin can colour exposed ammonia radicals. This allows FIGlu to be differentiated from glutamic acid by comparing two samples. However it has a poor sensitivity of 20-50 $\mu gm.ml^{-1}$ (Luhby and others 1959).

Other types of chromatography have been developed. High voltage electrophoresis uses 6000 volts with a current of 80 milliamperes to give a sensitivity of 10 $\mu gm.ml^{-1}$ (Knowles and others 1960). However, it is expensive, requiring specially constructed apparatus. In 1962, Knowles and Prankerd using a loading dose of 20 gm histidine showed that in 18 patients with both a presumed deranged folic acid metabolism and a vitamin B_{12} deficiency, only 11 had an increased urinary FIGlu excretion and with vitamin B_{12} treatment all but 1 of these returned to normal.

Conventional electrophoresis uses a lower voltage of 200-500 volts (Kohn and others 1961a, 1961b). Folic acid and vitamin B_{12} deficient patients were given a loading dose of 15 gm histidine and urine was collected for 5 hours. All folic acid deficient patients had increased FIGlu excretion. Similarly, 4 out of 15 vitamin B_{12} deficient patients also showed an increase. Because FIGlu separation from glutamic acid can be incomplete, conventional electrophoresis with an elution assay has been developed. FIGlu was converted to glutamic acid using

alkaline hydrolysis after electrophoresis and this is stainable by ninhydrin (Zalusky and Herbert 1962). Two samples, one having been hydrolysed, are assayed and the colours compared. Using this, patients were loaded with 20 gm oral histidine and their urine was collected over 12 hours in 2 ml concentrated HCL. Six controls excreted a mean of 19 mg of FIGlu compared to 561 mg in 8 patients with folic acid deficiency and 288 mg in 7 patients with vitamin B_{12} deficiency.

Knowles (1962) examined patients with steatorrhoea by giving them a 20 gm histidine loading dose. Using both high and conventional voltage electrophoresis, he showed that the high voltage technique was superior in detecting raised FIGlu excretions and confirmed that minor folic acid deficiency occurs with this disease. Additionally, he showed that FIGlu appears in the urine within 2 hours and peaks at 5-6 hours and is hardly present after 12 hours.

3) Enzymic Assay

If urine is incubated with THF and a semi-purified liver concentrate, 2 enzymes, THF transferase and cyclodeaminase, will convert FIGlu to 5,10-MethenylTHF (Tabor and Rabinovitz 1956). This undergoes spontaneous non-enzymatic conversion to 10-FormylTHF. Acidification converts it back to 5,10-MethenylTHF.

FIGlu Transferase
FIGlu + THF ----------> Glutamic acid + 5 FormiminoTHF

Cyclodeaminase
5 FormimnoTHF----------> 5,10-MethenylTHF + NH3

5,10-MethenylTHF + OH^- <----------> 10-FormylTHF + H^+

Because 5,10-MethenylTHF has a peak absorption at 350 mμ, its concentration can be found spectrophotometrically using standard solutions (Tabor and Wyngarden 1958). This method is sensitive taking only a relatively short time. However, production of the enzymes is both time consuming and difficult. The solution is often impure containing contaminants including histidinase and urocanase which will metabolise any urocanic acid or histidine. Therefore urinary FIGlu measurements will tend to be greater than the true amount. Carter and others (1961) investigated FIGlu excretion in patients with hepatic cirrhosis collecting 24 hour urine samples for 5 days. The urine was stored in 20 ml concentrated HCL. Subjects received no histidine on day 1, 15 gm in 3 doses on day 2, nothing on day 3 and 15 gm with 20 mg folic acid on days 4 and 5. Those with hepatic cirrhosis had raised FIGlu excretion which was unresponsive to folic acid.

Modifications of this assay have been described. Silverman and others (1958) used it combined with a microbiological assay. They enzymatically converted FIGlu to 10-FormylTHF and then ascorbic acid changed this to 5N-FormylTHF. This was measured using a microbiological assay performed simultaneously on both heated and unheated urine and FIGlu concentrations were calculated by subtracting the two results. However the test is time consuming taking over 36 hours and it has a poor specificity especially if the enzyme preparation is impure. However it

has a good sensitivity of 1-5 $\mu m.ml^{-1}$.

Chanarin and Bennett (1963) further modified this technique by measuring the amount of 5,10-N MethenylTHF that had been converted from 10-N FormylTHF by acidification. Two assays were done on heated and unheated urine. The test can be performed in a day, but it is only 20% as accurate as the method of Tabor and Wyngarden and has the same problems as the Silverman assay.

4) Chemical Assay

Two methods have been used. Alkaline ferricyanide-nitroprusside turns red with reducing agents. Whilst FIGlu will reduce it (Tabor and Wyngarden 1958), so will many other urinary compounds (histidine, ascorbic acid, etc) and so it has a poor specificity. Its main advantage is speed because the reaction is rapid and the colour intensity can be found immediately. Naphthoquinone-4-sulphonate turns reddish-brown with imino compounds. Again, it has a poor specificity because it can also react with amino acids. Because it reacts with FIGlu it can be used instead of ninhydrin to identify FIGlu in a chromatography assay.

In conclusion, the most sensitive method of measuring FIGlu is enzymatic, although it can be inaccurate especially if the enzyme preparation is impure. FIGlu is unstable in urine with a pH greater than 5 (Luhby and others 1959) and it rapidly decomposes to glutamic acid at room temperature and even at -20°C can only be

kept for a short time. However at pH's of 2 or less it remains stable even at room temperature for months. Addition of 1ml concentrated HCl per 100 ml urine will reduce the pH to below 2 and therefore preserve FIGlu (Chanarin and Bennett 1962, Carter and others 1961, Zalusky and Herbert 1962).

There is no definitive test procedure. One recommended method is to give 15 gm oral histidine monochloride, and collect all urine for the next 8 hours. The patient empties his bladder before ingestion and urine is then collected in 5 ml concentrated hydrochloric acid (British Medical Journal 1969).

FIGlu excretion in 94 healthy people without histidine loading was measured using the modified enzymatic method of Tabor and Wyngarden (Rosenauerova-Ostra and others 1976). Sex had an insignificant effect but short term calorie restriction did decrease FIGlu excretion. Age had a small difference, with the 40 - 50 age group excreting slightly more FIGlu than either younger or older subjects.

Other diseases may increase FIGlu excretion. Liver disease (Carter and others 1961, Nigro 1967) and sarcoidosis increase it, probably due to a reduction in formimino-transferase enzyme concentration (Kohn and others 1961b). Patients with iron deficient anaemia also have raised excretion (Chanarin and others 1962).

What is the significance of this test? Examples have been given demonstrating its usefulness in detecting a functional deficiency of folate (Kohn and others 1961,

Chanarin and others 1962). This test does not assess total folate deficiency, only that of one co-enzyme, THF. When folate concentrations are normal, but urinary FIGlu excretion is increased, the test suggests an abnormality of folate metabolism associated with insufficient THF.

This test may be used to investigate the toxic effects of N_2O on vitamin B_{12}. N_2O will decrease THF concentrations and so increase urinary FIGlu excretion. This test is different to the other assays because it examines the intermediate phase between MS inhibition and marrow depression. It is therefore more sensitive than marrow or blood examination and because folate metabolism is important, abnormalities in it are more clinically relevant than MS inhibition. Therefore, this test has both increased sensitivity and clinical relevance to the toxic effect of N_2O. A further advantage is that it is relatively non-invasive, requiring only the ingestion of an amino-acid and the collection of urine.

The assay has previously been used to investigate N_2O toxicity in 3 studies. Rats were exposed to 50% N_2O in oxygen for 24 hours and their urine collected. Prior to exposure, one group was given 250 μmol L-methionine in 0.5 ml saline and the other group saline alone. Two extra control groups exposed only to air excreted no FIGlu. The methionine N_2O exposed rats excreted a mean of 36.5 (SE 6.7) μmol FIGlu compared to 52.2 (SE 7.5) μmol in the N_2O saline group (Deacon and others 1983). Therefore, rats exposed N_2O have an increased FIGlu excretion even if no

histidine is given. Methionine decreased the amount excreted as was expected (Silverman and Pitney 1958, Herbert and Sullivan 1963).

A recent study examined FIGlu excretion in rats of different ages (Koblin and Tomerson 1990). The urine of young, middle aged and old rats exposed to 60% N_2O for 6 hours was collected. All had increased FIGlu excretion on the day after exposure, returning to normal on the second day. The older the rat, the greater the excretion.

It is possible that N_2O could affect FIGlu transferase enzyme and this could cause increases in FIGlu excretion although exposure of rats to 50% N_2O for 10 days had no effect on its activity (Perry and others 1980) suggesting that increases in FIGlu occurs due to an effect on folate metabolism.

Hawkins and others (1987) assessed FIGlu excretion in 6 healthy volunteers anaesthetised with 70% N_2O in oxygen at 2 atmospheres (a total of 2.2 - 4.9 atm.hours N_2O exposure which is equivalent to 70% N_2O for 3.1 to 7.0 hours). They were given a histidine load daily for 2 days before and several days after exposure and urine was collected every 8 hours. FIGlu was converted to glutamate and this was assayed enzymatically. Two subjects had an increase in FIGlu excretion after exposure. The study did not state the sensitivity of the method used to measure FIGlu or the dosage of histidine given. In addition, it is difficult to extrapolate this result to a clinical situation because it is possible that the added stress of surgery may have a cumulative effect on N_2O toxicity.

Cancer and folate deficiency

Folate deficiency is associated with malignant disease (Chanarin 1979) for several reasons including poor dietary intake and increased folate utilisation by malignant cells (Saleh and others 1982). Another reason may be impaired synthesis of folate enzymes (Davis 1986).

The serum folic acid activity in patients with both leukaemia and cancer has been found using the L.casei assay. In controls it is 2.5 to 7.5 (mean 4.6) $\mu g.ml^{-1}$ whilst in malignant tumours is 1.1 to 8.5 (mean 2.3) $\mu g.ml^{-1}$ and in leukaemias 0.8 to 7.0 (mean 2.7) $\mu g.ml^{-1}$. Overall, it is significantly lower in patients with malignancies (Rama Rao and others 1965). Therefore it appears that cancer has a deleterious effect on folate activity. Measurement of serum and red blood cell folate concentrations indicated they were normal in patients with localised cancer, but low if there was metastatic disease (Magnus 1982).

Does this abnormal folate activity cause an increased excretion of FIGlu? Seventy patients with neoplastic disease were loaded with 20 gm oral histidine and all urine produced for 8 hours collected. FIGlu concentrations were found by converting it to glutamate by heat and using electrophoresis. Controls excreted 0 - 30 (mean 10.5, SD 9.8) μmol whilst patients with malignancy excreted up to 25 times more with 49% excreting concentrations greater

than 30 μmol (mean 66, SD 118). Ten patients had cancer of the lung and of these, 5 had raised excretions. One had cancer of the oesophagus and a raised excretion (Carey and others 1964). A similar increase in FIGlu excretion was seen in patients with carcinoma (Noeypatimanond and others 1966). It therefore appears that many patients with malignancy have raised FIGlu excretion and have abnormalities of folate metabolism.

Are patients more susceptible to N_2O if they have a malignancy? It is possible because N_2O effects on the folate pathway may be additive. The uptake and utilisation of the various folate co-enzymes by tumour cells is similar to that of rapidly replicating cells. Therefore individuals with cancer exposed to N_2O may have similar effects to patients with rapidly growing tissues (pregnant women, young children, patients recovering from trauma, etc). These groups of patients are ethically more difficult to study and it may be possible to extrapolate results from cancer patients to them. This maybe a simple method of assessing the possible dangers of N_2O on these types of individuals.

Thesis experiments

This thesis uses the FIGlu test to examine the possible toxicity of N_2O inhalation that may occur in a variety of situations. A suitable FIGlu assay system has been developed with a standardised histidine loading and urine collection system. Five groups have been examined;

1. Normal subjects, not exposed to N_2O

2. Subjects receiving a N_2O anaesthetic for limb surgery

3. Subjects not receiving a N_2O anaesthetic for limb surgery

4. Subjects with oesophageal and bronchial carcinoma receiving a N_2O anaesthetic

5. Anaesthetists exposed chronically to N_2O pollution.

The first group was studied in order to find normal excretion values and to compare these to other studies. The second group examines the effects of N_2O anaesthesia upon patients to see if there is a possible undetected toxic action. Group 3 acted as controls for group 2 to ensure that surgery itself had no effect. Group 4 was studied in order to examine a high risk group and see if there was an increase in excretion greater than that seen in normal risk patients. Finally, group 5 was used to assess the risk to anaesthetists who were exposed to N_2O in their everyday work.

From these experiments, it was hoped to obtain a better understanding of the possible toxicity of N_2O on the vitamin B_{12}/folate metabolic pathway in a variety of conditions were N_2O might be given.

CHAPTER FIVE

METHODOLOGY

Each of the studies described in this thesis has received full approval from the local Anaesthetic Ethics Committee. All subjects gave full informed consent and all were told of their right to withdraw.

The description of the methods used is in three sections. Firstly, the assay system developed for measuring urinary FIGlu concentration in the urine will be described. The second section will explain how the formiminoglutamic acid (FIGlu) urine excretion test was performed and the third part will define the experiments on the different subject groups.

THE FIGlu URINE EXCRETION TEST

A. ASSAY SYSTEM

The most accurate and simplest method of measuring urinary FIGlu is the spectrophotometric enzymatic method of Tabor and Wyngarden (1958) because all the other techniques have problems. Some take too long to complete, some require expensive equipment and others are insufficiently sensitive to detect small amounts of FIGlu. The enzymatic method has been modified because pure enzymatic solutions are now commercially available. Tabor and Wyngarden in their description of the enzymatic method obtained the FIGlu converting enzymes from purification of homogenised hog liver. However, this method is complicated, time consuming and produces an impure

preperation with many contaminants which include urocanase and histidinase. These decrease the overall assay specificity because any histidine or urocanic acid that is present will be converted to FIGlu and then measured. Therefore, a pure enzyme preperation was used in these experiments. Other chemicals required included THF and FIGlu.

1. Enzymes. The two converting enzymes, FIGlu transferase and formiminoTHF cyclodeaminase can be bought in pure form (Sigma Chemicals). The enzymes are stored as crystals and when needed were dissolved in a buffer solution (1 M K2HPO4, pH=7.2). The activity of this solution can be changed by altering either enzyme concentration or the duration of the reaction. The standard reaction rate used in the experiments was that obtained when 100 μl of enzymatic solution reacted with the standard reagents described below to give an optical density reading of 0.8 - 1.2 when a known FIGlu concentration of 0.1 μmol.ml^{-1} was added. The enzymatic concentration giving this absorbency was found to be 25 IU.100 ml^{-1} if the reaction time was for 4 hours.

2. Tetrahydrofolic acid. A tetrahydrofolic acid (THF) solution was obtained by dissolving 88 mg of pure THF (Sigma Chemicals) in 18 ml 1.1 M mercaptoethanol (to prevent THF being oxidised) and 2 ml 0.2 N potassium hydroxide. This solution has a concentration of 4.4 mg or approximately 7-10 μmol.ml^{-1} THF. It retains its activity

for over a year if frozen at -20°C in the dark. THF assay reagent was made by adding 10 ml of this THF solution to 10 ml of buffer (1 M K2HPO4, pH=7.2) and 35 ml of distilled water. This volume is sufficient for 100 tests.

3. Formiminoglutamic acid. FIGlu (Sigma Company) was made up into 8 different concentrations in distilled water. Five were used as standard concentrations to draw a standard concentration/optical density curve. The other 3 were made into pools for testing the reproducibility of the different assay batches. The solution is highly stable if frozen at -20°C. The 5 concentrations used for the standard curve were 0.02, 0.05, 0.1, 0.15 and 0.2 $\mu mol.ml^{-1}$.

4. Urine samples were prepared for the assay by thawing batches overnight, fully mixing each sample by agitation and then centrifuging them at 6,000 rpm for 10 minutes to precipitate any solid material. The supernatant was pipetted off.

5. Assay system. Each sample was assayed in duplicate and the mean absorbency found. If the difference in absorbency of any 2 samples was greater than 10% they were re-assayed. Each sample (including standards, pools and urine) was assayed twice, once with enzyme added (sample)

and once with distilled water added (control). Subtracting the absorbancy values allowed for any differences in sample absorbency due to urine, etc. All samples were placed in 3 ml test tubes. In addition to all these samples, the absorbency for the enzyme (enzyme control) was found using enzyme blanks (no test sample added). Each test tube had the following solutions added (in μl).

Solution	Standard + urine		Enzyme Blanks	
	Sample	Control	Sample	Control
Reagent water+THF+buffer	550	550	550	550
Distilled water	-	250	100	350
Enzyme solution	250	-	250	-
Test sample urine, pool, std	100	100	-	-
Total volume	900	900	900	900

The samples were agitated to ensure good mixing and left in a warm (25°C) dark place for 4 hours. Then, 300 μl of 10% perchloric acid was added to stop the reaction and the samples left for a further 2 hours, completely mixed and the absorbency of each sample found using an ultraviolet spectrophotometer at an absorbency of 350 μm. Quartz curvettes were used for the samples. Calculation of

the concentration of FIGlu present in each sample was as follows.

1. Add each duplicate sample and divide by 2

2. Calculate enzyme blank absorbency;

   Enzyme sample - enzyme control

3. Subtract 2 from all other values

4. Subtract for each standard and urine sample;

   Sample - control

5. Plot standard curve for the 5 known FIGlu concentrations

6. Read other values off curve

The total FIGlu excretion for each urine sample was found by multiplying the concentration of the FIGlu (μmol.ml^{-1} urine) by the volume of urine collected (ml).

The linearity of the assay extended beyond the working range required, and the absorbency of a standard FIGlu solution agreed well with the calculated target value. The within-assay and between-assay coefficients of variation were 8% and 10% respectively.

B. THE FIGLU EXCRETION TEST

1. Histidine

Histidine was obtained in a pure crystalline form as histidine monochloride. One gram contains 0.74 gm

L.histidine. The oral histidine loading dose used was 10 gm histidine monochloride (= 7.4 gm histidine).

Because it is sparingly soluble in cold water and requires stirring to fully dissolve it, warm water was used. However, it has a particularly unpleasant taste although dissolving it in 50 ml orange juice masked this. The subjects swallowed it in one mouthful immediately after it had dissolved. The hospital subjects swallowed the histidine in the morning. All subjects received oral histidine on each consecutive day of the study.

2. Urine collections

Urine was collected in large (2 litre) plastic containers to which 5 ml concentrated hydrochloric acid had been added. The bladder was emptied after histidine ingestion and any urine produced over the next 8 hours was collected with the bladder being emptied at this time. The volume of each specimen was measured and aliquots taken and stored at -30° C for analysis later.

EXPERIMENTAL DESIGN

There were 5 sets of experiments on 5 different groups of subjects. All subjects were aged between 18 and 65 and all were ASA grade I and II. None had any evidence

of hepatic or renal disease. All had full biochemical and haematological testing which included urea and electrolytes, liver function tests, blood proteins, creatinine, haemoglobin, platelet and leukocyte counts with a blood picture, folate (plasma and red blood cell) and vitamin B_{12} concentrations. The 5 groups were;

1. Ten volunteers who were not exposed to N_2O were studied over 5 consecutive days. None had been exposed to N_2O for at least 1 month. This was the control group and normal FIGlu excretion for this experimental system was established.

2. Fifty subjects undergoing general anaesthesia for at least 60 minutes for limb surgery. These subjects were studied over 6 days. The initial FIGlu excretion was found on the day before surgery and continued on the day of surgery and for the next 4 days. Full haematological investigations were carried out on the 1st and 4th post-operative days. Twenty mg temazepam was given orally as pre-medication 1 hour before induction, which was with approximately 4 $mg.kg^{-1}$ thiopentone and 70 mg suxamethonium for tracheal intubation. Anaesthesia was maintained with 70% N_2O in oxygen, enflurane being given as required. The duration of N_2O inhalation was noted. Papaveretum (10-20 mg) was given intravenously and also used intramuscularly in the same dosage for post-operative

pain relief.

3. Five subjects receiving no N_2O for limb surgery of at least 60 minutes duration. They were studied in a similar manner as the subjects in group II, except instead of a general anaesthetic they received either a subarachnoid or epidural anaesthetic with 0.5% bupivacaine heavy or 0.5% bupivacaine plain respectively. Intra-operative sedation was with midazolam as required. Postoperative pain relief and investigation was as for group II.

4. Twenty subjects, 10 with oesophageal cancer (Group 4a) and 10 with bronchial cancer (group 4b) were studied. Both the timing and dose of histidine given were the same as for Group II. The method used for anaesthesia was standardised and consisted of a premedication of temazepam 20 mg with thiopentone 4 $mg.kg^{-1}$, suxamethonium 70 mg for induction. Anaesthesia was maintained using N_2O in oxygen, enflurane, morphine 10 mg and an atracurium infusion of 0.5 $mg.hour^{-1}$. Up to 70% N_2O was given depending upon the patient's arterial oxygenation. Total N_2O exposure (calculated as hours of 70% N_2O) was noted. Postoperative pain relief was provided by a morphine infusion of 1 $mg.hour^{-1}$ with an extra 2.5 mg IM as needed. Histidine was given daily down the nasogastric tube. Subjects undergoing thoracotomy for lung carcinoma received a

similar anaesthetic.

5. Ten anaesthetists exposed to N_2O pollution during their normal work were studied. All were male, aged 24 to 31 and had worked as full time anaesthetists for at least 6 months. They were all healthy. They were studied over 7 consecutive days which included a normal working week and a weekend when they were not in theatre. The mean concentration of each anaesthetist's daily exposure to N_2O was found using personal passive diffusive samplers. These allow N_2O to enter but not leave and they provide a time weighted average exposure (Gray 1989) when worn on the lapel throughout theatre exposure. At the start of the list, they were uncapped and then recapped at the end. The time that they were open for was recorded. Measurement of the N_2O they contained was by gas chromatography.

CHAPTER SIX

RESULTS

Group 1.

Subject data for age, weight and sex for group 1 are shown in table II. The mean age of group 1 was less than that of the other groups (Students unpaired t test)(Table VI). All 10 control subjects who had had no N_2O exposure had normal biochemical and haematological results. The mean red blood cell and plasma folate concentrations were 452.6 and 4.3 $ng.ml^{-1}$ (SD 168 and 1.2 $ng.ml^{-1}$) respectively. The mean vitamin B_{12} concentration was 557 $pg.ml^{-1}$ (SD 170 $pg.ml^{-1}$). All were within normal limits.

All the control subjects excreted formiminoglutamic acid (FIGlu) on each of the 5 days (Table VII, Fig.5). The total mean excretion for all 5 days (50 samples) was 26.99 μmol (SD 21.95, 95% CI 20.8-33.3). However, excretion varied greatly ranging from 0.33 to 74.32 μmol. The mean daily total excretion of FIGlu for all the subjects was similar on each of the 5 days (ANOVA). Because no cumulative effect was seen, it indicates that the histidine loading dose used in these experiments did not saturate the catabolic system when given for 5 days (Table XVII). The total mean excretion of each individual subject compared to the others was the same (ANOVA).

Group 2.

Sixty six patients who received a N_2O anaesthetic for limb surgery were studied. Sixteen were withdrawn from the trial; 9 due to severe nausea postoperatively preventing histidine ingestion, 4 due to errors in urine collection, 1 due to early hospital discharge, 1 because the patient required re-operation and 1 due to failure to prescribe histidine correctly. Subject data is in table III. The subjects wre older than the control group 1 subjects (Students unpaired t test, $p<0.001$, Table VI). The other groups are comparable to group 2.

The 50 subjects who completed the trial had the following operations; 17 total hip, 11 elbow and 15 knee replacements, 5 ankle and foot operations and 2 removal of infected plates. Duration of N_2O inhalation was from 64 to 312 minutes with a mean of 132.3 minutes (SD=56.5)(Fig.6).

Pre-operative biochemical and haematological screening was normal for all patients except in one individual who had a low vitamin B_{12} concentration (194 μmol with normal > than 220). Blood samples on the 1st and 4th postoperative days were all normal with no evidence of marrow depression. All urine samples contained FIGlu (Table VIII-XIII, Fig 7). On the preoperative and operative days (days 1 and 2) all patients except for a different individual on each day excreted FIGlu within the control range (Tables VIII and VIIII). Mean FIGlu excretion for these two days was 24.8 (SD 22.3, 95% CI 18.4-31.1) and 28.9 (SD 23.6, 95% CI 22.2-35.6) μmol

respectively (Table XVII). These concentrations are similar to those found from control group 1.

FIGlu excretion was increased on the first postoperative day (day 3) with 22 patients excreting more than 69.8 μmol (i.e. the mean + 2SD those of the control group)(Table X). There was a statistical difference between amounts excreted on this day and those in the control group 1 (unpaired Students t test $p<0.0001$). The range of FIGlu excretion was great with a mean of 78.21 (SD 67.38, 95% CI 52.7-103.7) μmol (Table XVII). There was a decrease in the number of high FIGlu excretors on the next day (day 4) with 12 individuals excreting more than 70 μmol (Table XI). There was still a statistical difference between this and control values ($p<0.0001$ with t test). Mean excretion was 61.52 (SD 50.98, 95% CI 42.2-80.8) μmol (Table XVII).

FIGlu excretion returned to that for the pre-operative controls on the following two days (days 5 and 6) with 3 and no subjects respectively excreting more than 70 μmol (Tables XII and XIII). The means (SD, 95% CI) were 25.49 (21.11, 22.4-37.2) and 29.80 (18.92, 24.1- 39.5) μmol (Table XVII).

The patients who excreted FIGlu in greater amounts were exposed to N_2O (mean 158.6, SD 64.6 minutes) for longer than normal patients (mean 113.3, SD 44.7 minutes)(Students unpaired t test, $p<0.008$). No patient exposed to 70% N_2O for less than 90 minutes had an increase in FIGlu excretion whilst all exposed for more than 211 minutes had increased excretions. For inbetween

exposures, a similar number of individuals had raised and normal excretions (Fig 8).

The patient with a low vitamin B_{12} concentration had normal FIGlu excretion throughout. The 22 subjects with a FIGlu excretion of greater than 70 μmol on day 3 had similar plasma and red blood cell folate and vitamin B_{12} concentrations when compared to the lower group.

Group 3.

The local anaesthetic group had 3 total hip and 2 knee replacements. Subject data is in table IV; they were older than the control group 1 (Students unpaired t test, $p<0.001$) but were similar to the other groups. All had normal biochemical and haematological results. Surgical duration was similar to that of group 2 and it lasted for a mean of 111.4 minutes (SD=33.1).

Again all excreted FIGlu daily (Table XIV, Fig 9). Excretion on the 1st day was similar to the control group 1 subjects and it stayed within this range over the next 5 days (t test comparison to control excretions)(Table XVII). There was no statistical increase in excretion on any of the study days including day 3 and 4 (ANOVA).

Group 4.

Twenty patients were studied. Ten in group 4a underwent operations for lung tumour (7 adenocarcinoma and 3 with oat cell carcinoma) and 10 in group 4b underwent resection for oesophageal tumour. There were no withdrawals from the trial and all had a thoracotomy. Patient data is in Table V. The ages were greater than group 1 (Table XVII) but were comparable to the other groups. Weights were slightly less than other groups, but not statistically significant. Preoperative haematology, plasma folate, red blood cell folate and vitamin B_{12} concentrations were all normal. Biochemistry was normal in group 4a whilst in group 4b, there was a non-significant reduction in the concentration of total plasma proteins ($0.71 gm.ml^{-1}$) and albumin ($0.34 gm.ml^{-1}$).

All excreted FIGlu on each of the 6 days (Tables XV and XVI). Excretion of FIGlu for each of these 2 groups was similar on each day (Students unpaired t test)(Fig. 10, 11). Whilst mean total excretion (41.8, SD 26.2, 95% CI 29.5-54.0) for both groups on day 1 was raised when compared to group 1, it was not statistically significant (Table XVII). Similar excretions were seen on day 2. In a similar manner to group 2, there was an increase in excretion on the next 2 days the mean being 60.4 (SD 43.8, 95% CI 40.0-81.0) and 53.1 (SD 35.1, 95% CI 36.7-69.6) respectively. There is no statistical significance in these increases. On day 3, 4 individual (2 in both groups) excreted significantly more (mean + 2SD) than on day 1.

Similarly for day 4 with 4 individuals, 3 being the same as for day 3 (1 in group 4a and 3 in group 4b). The excretion returned to day 1 levels on day 5 and day 6 (mean, SD and 95% CI respectively 36.8,22.7, 26.2-47.4 and 37.1,21.5, 27.0- 47.2).

The duration and concentration of N_2O exposure varied with the operations. Oesophageal surgery took longer than lung surgery with a calculated exposure to 70% N_2O of 154 (SD 42) minutes when compared to 96 (SD 32) minutes for lung surgery. Three patients undergoing lung surgery received small amounts of N_2O (12, 18 and 24 minutes of 70% N20). All had normal excretion on each of the 6 days. All of the 3 individuals with greatly increased FIGlu excretions were exposed to N_2O for longer periods (172, 164 and 173 minutes).

Group 5.

All anaesthetists had normal biochemical and haematological values. Table XIX shows the time weighted exposure of each of the anaesthetists for each day of the working week. The mean exposure of each individual ranged from 53.4 to 159.2 ppm. Four had no exposure to N_2O on one day of the experiment due to work with only local anaesthesia or due to a study day.

Fig. 12 and Table XVIII shows the amount of FIGlu excreted in the urine by each anaesthetist for each of the 7 days. The mean daily FIGlu excretion of all the anaesthetists for each of the 7 days is similar to that of the control group. There was no significant difference in any of the days mean total excretion (ANOVA). The anaesthetists all excreted similar quantities of FIGlu compared to each other.

Figure 5. Formiminoglutamic acid excretion (μmol)in 10 control individuals (group 1) over 5 consecutive days.

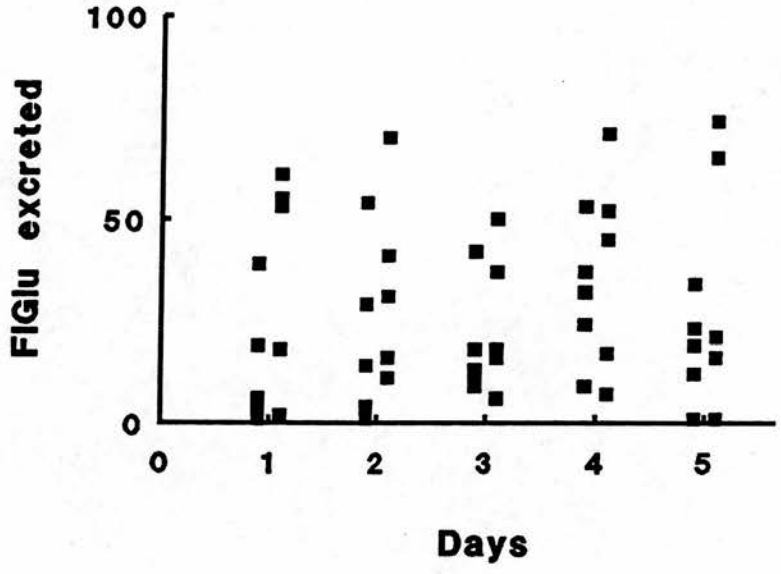

Figure 6. Duration (minutes) of inhalation of 70% nitrous oxide in 50 surgical patients (group 2).

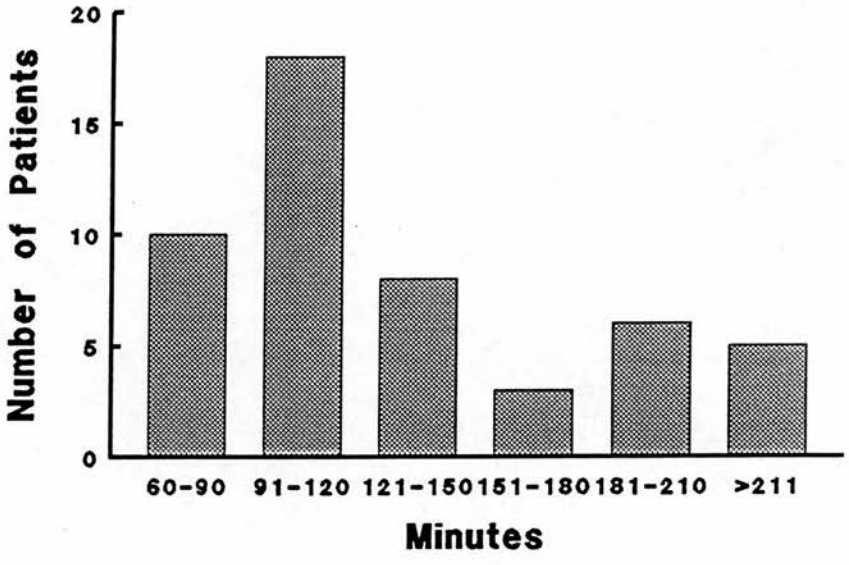

Figure 7. Formiminoglutamic acid excretion (μmol) in 50 surgical patients (group 2) over 6 consecutive days starting the pre-operative day.

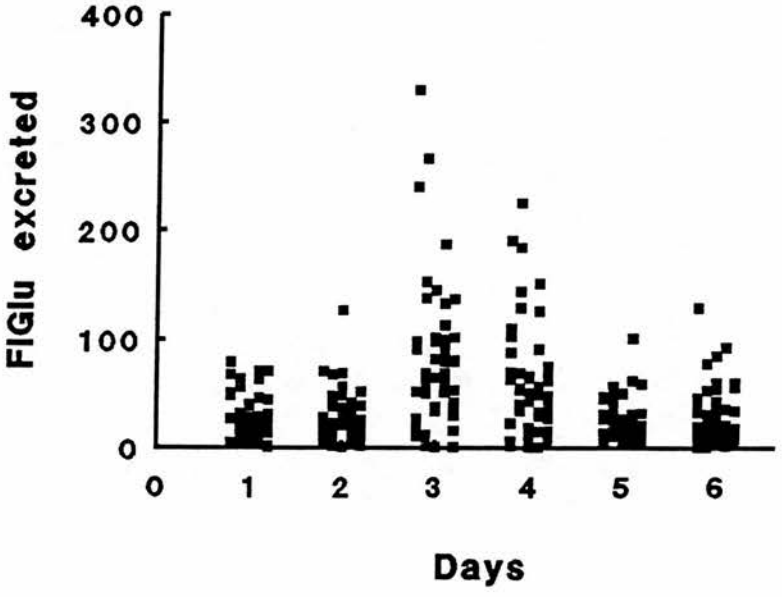

Figure 8. Raised (>70μmol) and normal (≤70μmol) formiminoglutamic acid excretion related to duration of 70% nitrous oxide.

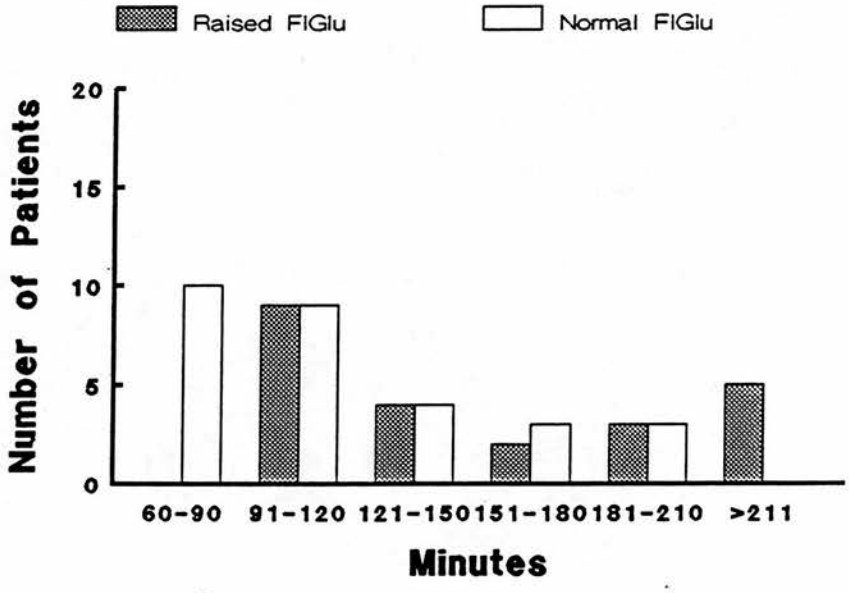

Figure 9. Formiminoglutamic acid excretion (μmol) in 5 surgical patients not exposed to nitrous oxide (group 3) over 6 consecutive days starting the preoperative day.

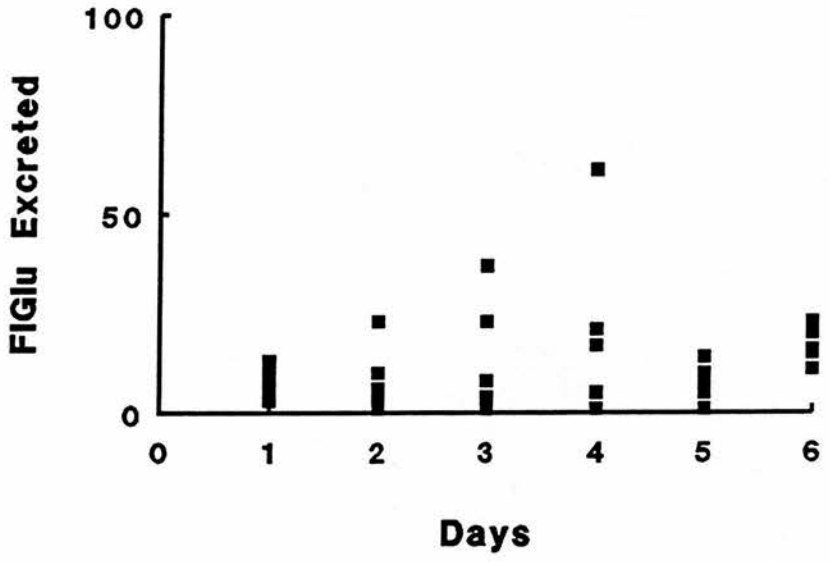

Figure 10. Formiminoglutamic acid excretion (μmol) in 10 patients undergoing thoractomy for lung carcinoma (group 4a) over 6 consecutive days starting the preoperative day.

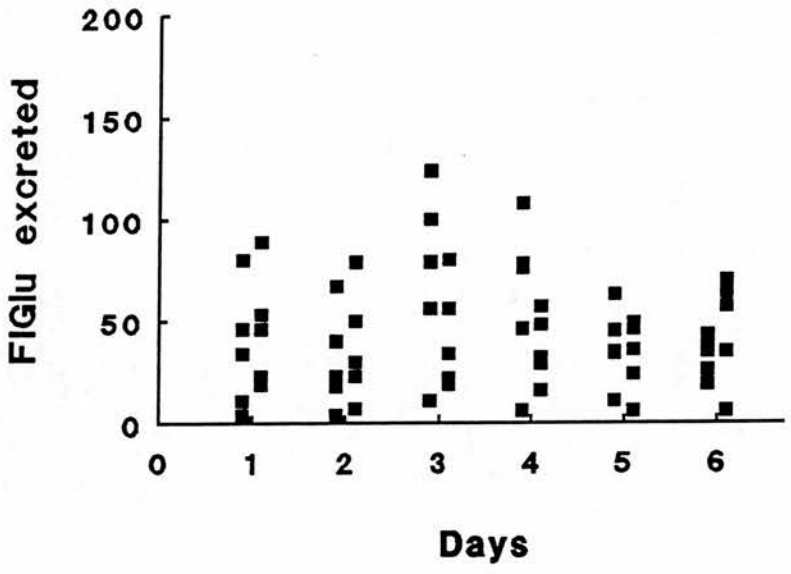

Figure 11. Patients undergoing thoracotomy for oesophageal carcinoma (group 4b) over 6 consecutive days starting the preoperative day.

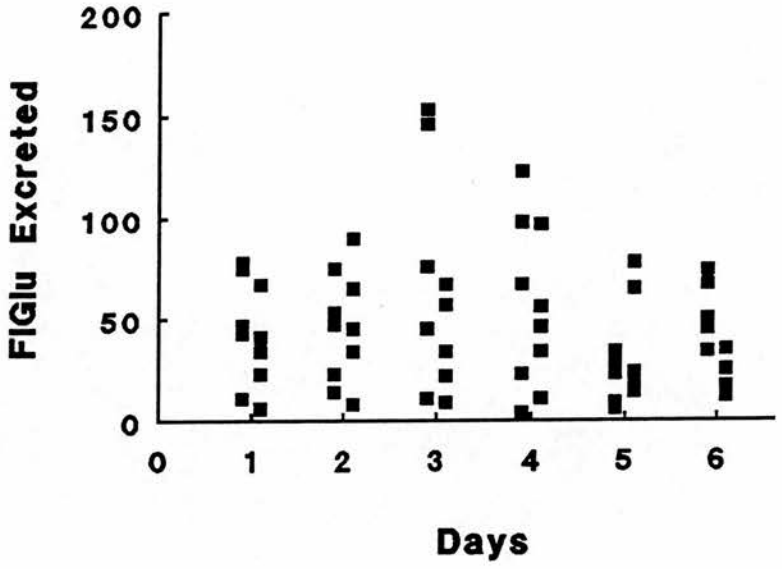

Figure 12. Formiminoglutamic acid excretion (μmol) in 10 anaesthetists over 7 consecutive days.

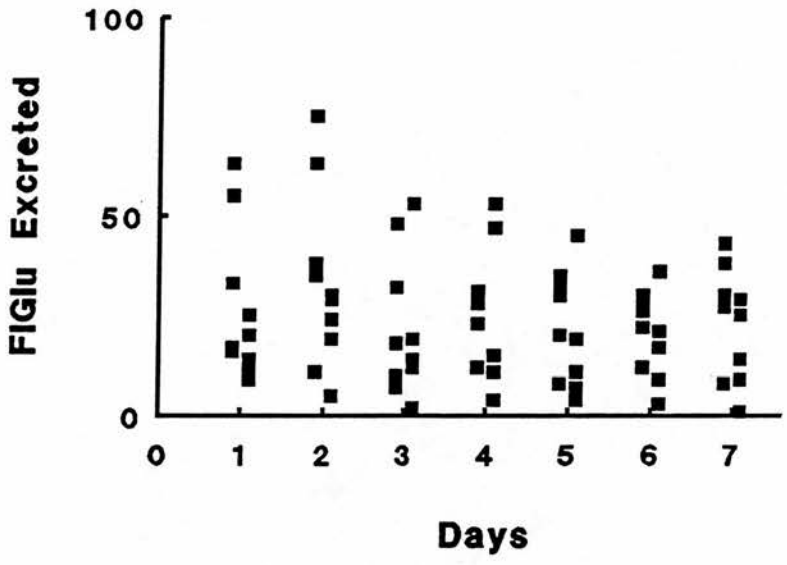

Table II. Group 1. Age (years), weight (Kg) and Sex.

	N=10
Age;	41 34 25 35 28 26 27 29 30 26
weight;	82 64 63 75 64 68 87 73 79 58
Sex;	Male - 7
	Female - 3

Table III. Group 2. Age (years), weight (Kg) and Sex.

N=50

Age;	44 23 62 58 51 47 48 54 60 62 27 47 48
	65 62 58 42 64 63 47 28 64 55 52 47 51
	48 62 37 54 61 65 62 38 57 49 65 62 64
	44 47 64 58 62 28 51 56 38 64 57
Weight;	56 62 64 47 52 62 82 64 76 92 64 64 84
	42 62 74 75 74 84 48 57 74 61 86 92 74
	68 82 68 72 64 59 82 74 65 49 91 57 74
	61 74 54 49 76 74 67 73 69 70 65
Sex:	Male - 24
	Female - 26

Table IV. Group 3. Age (years), weight (Kg) and Sex.

N=5

Age;	54 47 74 78 78
Weight;	74 64 74 78 78
Sex;	Male - 2
	female - 3

Table V. Group 4. Age (years), weight (Kg) and sex.

Group 4a, n=10

Age;	55 48 65 63 61 59 53 64 57 60
Weight;	85 74 54 58 47 58 67 63 69 54
Sex;	Male - 5
	Female - 5

Group 4b, n=10

Age;	57 50 61 63 61 55 63 56 49 63
Weight;	75 64 49 54 58 52 65 73 71 49
Sex;	Male - 4
	Female - 6

Table VI. Combined subject data for groups 1, 2, 3 and 4. Mean (SD).

Group	Age(years)	Weight(Kg)	Sex
Group 1	30.1 (7.5)*	71.5 (9.7)	7 male
n=10			3 female
Group 2	51.7 (11.8)	69.0 (12.7)	24 male
n=50			26 female
Group 3	56.2 (5.1)	73.6 (9.7)	2 male
n=5			3 female
Group 4a	58.5 (5.3)	62.9 (11.2)	5 male
n=10			5 female
Group 4b	57.8 (5.3)	61.0 (10.0)	4 male
n=10			6 female

* significant at $p<0.001$ against the other groups (unpaired t test).

Table VII. Urine excretion (ml), FIGlu concentration (μmol.ml^{-1}) and 8 hourly total FIGlu excretion for each subject (μmol) for each of the 5 days tested for Group 1 subjects.

Subject 1

Day	1	2	3	4	5
Urine volume	701	1117	1036	1048	891
FIGlu conc.	0.079	0.037	0.049	0.050	0.073
Total FIGlu	55.4	41.3	50.8	52.4	65.0

Subject 2

Day	1	2	3	4	5
Urine volume	1187	753	946	729	668
FIGlu conc.	0.033	0.073	0.045	0.045	0.052
Total FIGlu	39.2	55.0	42.6	32.8	34.7

Subject 3

Day	1	2	3	4	5
Urine volume	420	350	410	390	420
FIGlu conc.	0.041	0.091	0.041	0.032	0.005
Total FIGlu	17.2	31.8	16.9	7.14	1.26

Subject 4

Day	1	2	3	4	5
Urine volume	410	480	330	420	360
FIGlu conc.	0.130	0.004	0.001	0.124	0.045
Total FIGlu	53.3	1.92	0.33	52.8	16.2

Subject 5

Day	1	2	3	4	5
Urine volume	365	300	525	410	360
FIGlu conc.	0.016	0.096	0.026	0.023	0.002
Total FIGlu	5.84	28.8	13.6	9.43	0.72

Subject 6

Day	1	2	3	4	5
Urine volume	280	340	380	400	320
FIGlu conc.	0.067	0.003	0.023	0.079	0.082
Total FIGlu	18.76	1.02	8.74	31.6	23.04

Subject 7

Day	1	2	3	4	5
Urine volume	841	971	1303	925	991
FIGlu conc.	0.073	0.063	0.029	0.077	0.075
Total FIGlu	61.4	70.9	37.8	71.2	74.3

Subject 8

Day	1	2	3	4	5
Urine volume	315	450	380	370	360
FIGlu conc.	0.005	0.024	0.048	0.047	0.059
Total FIGlu	1.57	10.8	18.24	17.39	21.24

Subject 9

Day	1	2	3	4	5
Urine volume	520	610	410	370	280
FIGlu conc.	0.002	0.019	0.015	0.145	0.043
Total FIGlu	1.04	11.59	6.15	53.65	12.04

Subject 10

Day	1	2	3	4	5
Urine volume	245	192	325	285	180
FIGlu conc.	0.011	0.076	0.036	0.087	0.106
Total FIGlu	2.69	14.59	11.7	24.79	19.08

Table VIII. Urine volume (ml) over 8 hours following histidine ingestion, FIGlu concentration ($\mu mol.ml^{-1}$) and total FIGlu (μmol) excreted for day 1 for all 50 patients undergoing limb surgery under a N_2O anaesthetic.

Subject	Urine Volume	FIGlu Conc	Total FIGlu	Subject	Urine Volume	FIGlu Conc	Total FIGlu
1	426	0.112	47.71	26	318	0.240	79.36
2	306	0.032	9.79	27	512	0.111	56.80
3	312	0.048	14.97	28	315	0.016	4.96
4	198	0.016	3.17	29	223	0.284	63.24
5	138	0.012	1.66	30	325	0.003	0.88
6	740	0.004	2.96	31	111	0.047	5.20
7	471	0.056	26.38	32	210	0.094	19.82
8	108	0.057	6.05	33	317	0.026	8.40
9	317	0.092	29.16	34	126	0.217	27.28
10	412	0.108	44.50	35	843	0.017	14.40
11	306	0.088	26.93	36	226	0.015	3.36
12	196	0.164	32.14	37	326	0.055	18.08
13	174	0.160	27.84	38	306	0.132	40.42
14	89	0.092	8.19	39	212	0.087	18.36
15	194	0.080	15.52	40	164	0.010	1.69
16	472	0.143	67.80	41	412	0.013	5.25
17	208	0.311	64.89	42	141	0.175	24.72
18	762	0.052	39.6	43	106	0.223	23.69
19	374	0.124	46.37	44	224	0.085	19.08
20	744	0.095	71.00	45	227	0.111	25.09
21	611	0.084	51.32	46	416	0.006	2.69
22	326	0.008	2.61	47	196	0.042	8.32
23	260	0.032	8.32	48	128	0.024	3.13
24	511	0.140	71.40	49	191	0.052	10.24
25	218	0.008	1.68	50	162	0.193	31.36

Table IX. Urine volume (ml) over 8 hours following histidine ingestion, FIGlu concentration ($\mu mol.ml^{-1}$) and total FIGlu (μmol) excreted for day 2 for all 50 patients undergoing limb surgery under a N_2O anaesthetic.

Subject	Urine Volume	FIGlu Conc	Total FIGlu	Subject	Urine Volume	FIGlu Conc	Total FIGlu
1	328	0.215	70.32	26	143	0.083	11.90
2	282	0.135	38.07	27	140	0.117	16.45
3	479	0.121	57.48	28	83	0.455	37.80
4	154	0.020	3.68	29	148	0.074	11.00
5	147	0.012	1.84	30	217	0.030	16.80
6	470	0.007	3.52	31	168	0.062	10.50
7	174	0.012	2.17	32	427	0.033	16.80
8	94	0.013	1.17	33	103	0.469	48.30
9	328	0.028	9.19	34	84	0.178	14.96
10	317	0.165	52,51	35	227	0.093	21.03
11	412	0.172	71.07	36	174	0.163	28.60
12	208	0.235	48.89	37	212	0.099	23.92
13	264	0.091	23.76	38	74	0.458	33.92
14	186	0.213	39.99	39	88	0.151	13.32
15	382	0.057	21.96	40	186	0.056	10.34
16	196	0.117	23.03	41	74	0.515	38.13
17	342	0.117	40.18	42	45	0.493	22.20
18	840	0.082	69.30	43	186	0.075	13.95
19	294	0.107	31.61	44	173	0.228	39.52
20	144	0.177	25.56	45	218	0.089	19.44
21	297	0.060	17.82	46	109	0.630	68.67
22	106	0.102	10.86	47	241	0.120	28.88
23	410	0.310	127.10	48	102	0.413	42.17
24	246	0.090	22.20	49	148	0.161	23.92
25	105	0.250	26.25	50	94	0.081	7.65

Table X. Urine volume (ml) over 8 hours following histidine ingestion, FIGlu concentration ($\mu mol.ml^{-1}$) and total FIGlu (μmol) excreted for day 3 for all 50 patients undergoing limb surgery under a N_2O anaesthetic.

Subject	Urine Volume	FIGlu Conc	Total FIGlu	Subject	Urine Volume	FIGlu Conc	Total FIGlu
1	414	0.221	91.94	26	327	0.015	52.22
2	194	0.045	8.69	27	423	0.028	11.97
3	319	0.454	144.92	28	420	0.157	65.85
4	254	0.525	133.32	29	346	0.291	100.92
5	268	0.061	16.29	30	326	0.247	80.51
6	860	0.022	19.26	31	186	0.529	98.31
7	102	0.012	2.17	32	303	0.230	69.70
8	72	0.019	1.38	33	227	0.150	34.16
9	416	0.272	113.15	34	90	0.563	50.68
10	404	0.251	102.50	35	475	0.089	42.35
11	916	0.262	240.36	36	194	1.706	330.88
12	416	0.368	153.01	37	172	0.295	50.80
13	574	0.144	82.65	38	174	0.221	38.52
14	204	0.394	80.29	39	65	1.456	94.65
15	411	0.195	80.23	40	94	0.008	0.83
16	308	0.086	26.61	41	274	0.086	23.46
17	172	0.358	61.64	42	189	0.296	56.11
18	183	0.196	35.77	43	224	0.170	38.12
19	790	0.237	187.10	44	124	0.682	84.58
20	822	0.163	134.12	45	74	0.418	30.95
21	394	0.037	14.74	46	222	1.151	255.62
22	924	0.150	138.97	47	322	0.034	10.89
23	328	0.300	98,66	48	74	1.382	102.30
24	486	0.129	60.35	49	82	0.893	69.61
25	198	0.183	36.28	50	152	0.349	53.04

Table XI. Urine volume (ml) over 8 hours following histidine ingestion, FIGlu concentration (μmol.ml^{-1}) and total FIGlu (μmol) excreted for day 4 for all 50 patients undergoing limb surgery under a N_2O anaesthetic.

Subject	Urine Volume	FIGlu Conc	Total FIGlu	Subject	Urine Volume	FIGlu Conc	Total FIGlu
1	528	0.210	110.89	26	418	0.150	62.73
2	324	0.161	66.98	27	318	0.168	53.55
3	142	0.468	66.46	28	519	0.036	18.60
4	238	0.384	91.39	29	323	0.090	29.07
5	1119	0.033	36.93	30	273	0.226	61.74
6	116	0.015	1.74	31	342	0.189	64.80
7	198	0.174	34.45	32	264	0.149	39.36
8	66	0.174	11.48	33	215	0.301	64.74
9	712	0.177	126.02	34	226	0.217	49.20
10	312	0.243	75.82	35	346	0.197	68.48
11	728	0.261	190.10	36	241	0.292	70.38
12	560	0.259	144.52	37	253	0.159	39.76
13	602	0.107	64.75	38	106	0.007	0.72
14	192	0.300	57.60	39	108	0.009	0.95
15	206	0.039	8.03	40	126	0.234	29.48
16	604	0.171	103.28	41	406	0.056	22.68
17	962	0.234	255.11	42	126	1.025	129.10
18	96	0.147	14.11	43	162	0.089	14.36
19	872	0.174	151.80	44	106	0.312	33.05
20	741	0.063	46.68	45	88	0.181	15.90
21	526	0.168	88.37	46	104	0.056	5.81
22	1044	0.177	184.82	47	108	0.511	55.20
23	174	0.252	43.84	48	62	0.826	51.19
24	343	0.161	55.08	49	224	0.071	16.00
25	254	0.106	27.00	50	184	0.265	48.81

Table XII. Urine volume (ml) over 8 hours following histidine ingestion, FIGlu concentration (μmol.ml^{-1}) and total FIGlu (μmol) excreted for day 5 for all 50 patients undergoing limb surgery under a N_2O anaesthetic.

Subject	Urine Volume	FIGlu Conc	Total FIGlu	Subject	Urine Volume	FIGlu Conc	Total FIGlu
1	142	0.051	7.24	26	264	0.102	26.91
2	416	0.065	21.22	27	189	0.114	21.60
3	78	0.117	9.13	28	261	0.028	7.27
4	147	0.216	31.78	29	419	0.035	14.82
5	570	0.012	11.11	30	259	0.031	8.16
6	108	0.079	8.95	31	264	0.173	45.75
7	412	0.101	41.41	32	156	0.365	56.94
8	194	0.101	19.50	33	324	0.085	27.45
9	890	0.114	101.46	34	104	0.614	63.89
10	74	0.084	6.22	35	295	0.073	21.68
11	308	0.153	47.12	36	238	0.190	45.24
12	512	0.069	56.06	37	128	0.189	24.22
13	192	0.028	5.47	38	212	0.026	5.50
14	104	0.045	4.68	39	121	0.247	29.89
15	316	0.048	15.17	40	205	0.036	7.07
16	344	0.150	5.16	41	282	0.050	14.10
17	382	0.029	10.89	42	104	0.301	31.30
18	348	0.145	50.63	43	108	0.052	5.62
19	174	0.150	26.10	44	214	0.035	7.45
20	367	0.180	6.61	45	221	0.081	17.97
21	109	0.099	10.79	46	392	0.079	31.16
22	374	0.049	18.51	47	74	0.659	48.78
23	478	0.108	51.62	48	109	0.196	21.21
24	357	0.059	21.00	49	278	0.043	12.01
25	328	0.100	32.55	50	372	0.159	59.20

Table XIII. Urine volume (ml) over 8 hours following histidine ingestion, FIGlu concentration ($\mu mol.ml^{-1}$) and total FIGlu (μmol) excreted for day 6 for all 50 patients undergoing limb surgery under a N_2O anaesthetic.

Subject	Urine Volume	FIGlu Conc	Total FIGlu	Subject	Urine Volume	FIGlu Conc	Total FIGlu
1	624	0.032	20.15	26	294	0.045	13.22
2	172	0.029	19.16	27	160	0.086	13.89
3	296	0.201	59.62	28	295	0.128	37.70
4	236	0.009	2.07	29	274	0.132	36.37
5	490	0.018	8.84	30	324	0.107	34.58
6	241	0.076	18.32	31	294	0.134	39.52
7	378	0.078	29.45	32	228	0.116	26.45
8	108	0.072	7.64	33	174	0.136	23.83
9	116	0.137	15.86	34	88	0.189	16.62
10	118	0.146	17.26	35	764	0.024	18.39
11	246	0.135	33.18	36	96	0.481	46.21
12	248	0.215	53.25	37	286	0.001	0.36
13	174	0.095	16.91	38	78	0.352	27.48
14	203	0.098	19.86	39	243	0.011	2.67
15	417	0.084	34.86	40	151	0.073	11.04
16	292	0.066	19.18	41	174	0.030	5.16
17	106	0.192	20.34	42	228	0.090	20.49
18	266	0.133	35.38	43	142	0.305	43.32
19	628	0.148	93.07	44	196	0.107	21.04
20	411	0.148	60.91	45	106	0.112	11.92
21	874	0.152	131.50	46	561	0.003	1.41
22	511	0.061	31.17	47	194	0.407	78.88
23	829	0.102	85.01	48	246	0.221	54.55
24	421	0.008	3.19	49	416	0.033	13.80
25	210	0.070	14.06	50	263	0.213	56.01

Table XIV. Urine volume (ml) over 8 hours following histidine ingestion, FIGlu concentration (μmol.ml^{-1}) and total FIGlu (μmol) excreted for day 6 for all 50 patients undergoing limb surgery without any exposure to N_2O.

Patient 1

Day	1	2	3	4	5	6
Urine volume	206	172	278	143	374	262
FIGlu conc.	0.050	0.060	0.016	0.428	0.038	0.077
Total FIGlu	10.39	10.37	4.48	61.20	14.14	20.24

Patient 2

Day	1	2	3	4	5	6
Urine volume	428	184	122	196	204	310
FIGlu conc.	0.006	0.018	0.308	0.003	0.022	0.075
Total FIGlu	2.52	3.33	37.52	0.68	4.59	23.13

Patient 3

Day	1	2	3	4	5	6
Urine volume	362	244	176	184	183	224
FIGlu conc.	0.023	0.011	0.003	0.112	0.052	0.050
Total FIGlu	8.38	2.70	0.46	20.57	9.72	11.24

Patient 4

Day	1	2	3	4	5	6
Urine volume	247	96	106	84	126	156
FIGlu conc.	0.051	0.240	0.225	0.200	0.062	0.105
Total FIGlu	12.65	23.02	23.87	16.82	7.85	16.44

Patient 5

Day	1	2	3	4	5	6
Urine volume	108	74	114	252	184	162
FIGlu conc.	0.060	0.018	0.072	0.021	0.002	0.095
Total FIGlu	6.49	1.31	8.23	5.38	0.42	15.42

Table XV. Urine volume (ml) over 8 hours following

histidine ingestion, FIGlu concentration ($\mu mol.ml^{-1}$) and total FIGlu (μmol) excreted for all 6 days 6 for all 10 patients undergoing thoracotomy for lung carcinoma.

Patient 1

Day	1	2	3	4	5	6
Urine volume	236	216	372	68	104	283
FIGlu conc.	0.142	0.108	0.269	1.154	0.233	0.068
Total FIGlu	33.62	23.48	100.18	78.52	24.32	19.34

Patient 2

Day	1	2	3	4	5	6
Urine volume	543	165	270	145	562	732
FIGlu conc.	0.148	0.112	0.294	0.527	0.063	0.049
Total FIGlu	80.44	18.60	79.50	76.45	35.76	35.58

Patient 3

Day	1	2	3	4	5	6
Urine volume	298	90	124	325	272	197
FIGlu conc.	0.038	0.448	0.454	0.143	0.166	0.221
Total FIGlu	11.24	40.30	56.32	46.72	45.30	43.67

Patient 4

Day	1	2	3	4	5	6
Urine volume	136	321	680	412	230	108
FIGlu conc.	0.033	0.112	0.182	0.263	0.274	0.245
Total FIGlu	4.58	67.42	124.30	108.46	63.10	26.50

Patient 5

Day	1	2	3	4	5	6
Urine volume	378	321	312	645	239	340
FIGlu conc.	0.122	0.037	0.036	0.010	0.049	0.011
Total FIGlu	46.43	6.84	11.36	6.50	11.64	37.83

Table XV(cont).

Patient 6

Day	1	2	3	4	5	6
Urine volume	492	95	234	120	356	563
FIGlu conc.	0.109	0.071	0.083	0.238	0.016	0.102
Total FIGlu	53.86	6.84	19.43	28.64	5.74	57.4

Patient 7

Day	1	2	3	4	5	6
Urine volume	731	246	544	870	675	432
FIGlu conc.	0.032	0.324	0.042	0.037	0.036	0.163
Total FIGlu	23.12	79.63	56.34	32.43	24.06	70.52

Patient 8

Day	1	2	3	4	5	6
Urine volume	340	345	628	89	934	328
FIGlu conc.	0.263	0.146	0.128	0.640	0.050	0.108
Total FIGlu	89.53	50.43	80.42	57.06	46.94	35.76

Patient 9

Day	1	2	3	4	5	6
Urine volume	586	85	211	152	105	328
FIGlu conc.	0.079	0.355	0.267	0.318	0.469	0.197
Total FIGlu	46.54	30.22	56.42	48.31	49.32	64.79

Patient 10

Day	1	2	3	4	5	6
Urine volume	98	169	143	328	462	296
FIGlu conc.	0.196	0.139	0.241	0.050	0.079	0.021
Total FIGlu	19.21	23.64	34.68	16.41	36.60	6.62

Table XVI. Urine volume (ml) over 8 hours following histidine ingestion, FIGlu concentration ($\mu mol.ml^{-1}$) and total FIGlu (μmol) excreted for all 6 days 6 for all 10 patients undergoing thoracotomy for oesophageal carcinoma.

Patient 1

Day	1	2	3	4	5	6
Urine volume	124	243	295	179	325	204
FIGlu conc.	0.351	0.096	0.153	0.132	0.106	0.169
Total FIGlu	43.62	23.54	45.32	23.68	34.59	34.50

Patient 2

Day	1	2	3	4	5	6
Urine volume	90	78	216	328	281	295
FIGlu conc.	0.873	0.607	0.325	0.281	0.080	0.228
Total FIGlu	78.64	47.42	153.64	123.54	22.75	67.42

Patient 3

Day	1	2	3	4	5	6
Urine volume	384	253	450	348	240	370
FIGlu conc.	0.121	0.211	0.324	0.281	0.281	0.007
Total FIGlu	46.86	53.54	146.21	98.43	67.54	2.63

Patient 4

Day	1	2	3	4	5	6
Urine volume	146	452	327	683	496	257
FIGlu conc.	0.076	0.032	0.233	0.098	0.017	0.177
Total FIGlu	11.20	14.75	76.54	67.54	5.87	45.63

Patient 5

Day	1	2	3	4	5	6
Urine volume	354	642	342	784	684	356
FIGlu conc.	0.212	0.117	0.033	0.004	0.013	0.209
Total FIGlu	75.43	75.32	11.43	3.76	8.90	74.54

Table XVI (cont)

Patient 6

Day	1	2	3	4	5	6
Urine volume	625	348	624	851	254	573
FIGlu conc.	0.054	0.260	0.054	0.067	0.308	0.026
Total FIGlu	33.65	90.54	34.21	56.78	78.43	14.74

Patient 7

Day	1	2	3	4	5	6
Urine volume	365	257	356	852	80	106
FIGlu conc.	0.112	0.029	0.161	0.040	0.842	0.378
Total FIGlu	41.13	7.54	57.64	34.75	67.43	40.11

Patient 8

Day	1	2	3	4	5	6
Urine volume	90	172	140	350	285	284
FIGlu conc.	0.262	0.201	0.161	0.133	0.085	0.124
Total FIGlu	23.64	34.67	22.65	46.71	24.54	35.43

Patient 9

Day	1	2	3	4	5	6
Urine volume	274	739	194	375	108	384
FIGlu conc.	0.021	0.088	0.347	0.257	0.604	0.033
Total FIGlu	5.87	65.32	67.32	97.42	65.32	12.84

Patient 10

Day	1	2	3	4	5	6
Urine volume	605	860	475	382	639	274
FIGlu conc.	0.111	0.053	0.018	0.030	0.029	0.061
Total FIGlu	67.43	45.73	8.96	11.64	18.64	16.85

Table XVII. Mean, SD excretion of FIGlu on each of the study days for groups 1, 2, 3 and 4 (μmol).

Day	Group			
	1	2	3	4
1	25.6, 24.3	24.8, 22.3	8.1, 3.9	41.8, 26.2
2	26.8, 23.3	28.9, 23.6	8.1, 9.0	40.2, 25.7
3	20.7, 17.0	78.2, 67.3	14.2, 15.4	60.4, 43.8
4	35.2, 21.4	61.5, 51.0	20.9, 23.9	53.1, 35.1
5	26.8, 22.0	25.5, 20.3	7.3, 4.7	36.8, 22.7
6	- -	29.8, 26.0	17.3, 14.1	37.1, 21.5

Table XVIII. Urine volume (ml) over 8 hours following histidine ingestion, FIGlu concentration ($\mu mol.ml^{-1}$) and total FIGlu (μmol) excreted for 7 consecutive days in 10 anaesthetists exposed to N_2O in their normal working week.

Anaesthetist 1

Day	1	2	3	4	5	6	7
Urine volume	840	615	105	660	560	480	780
FIGlu conc.	0.008	0.072	0.060	0.026	0.048	0.050	0.040
Total FIGlu	6.72	44.28	6.30	17.16	26.88	2.40	31.20

Anaesthetist 2

Day	1	2	3	4	5	6	7
Urine volume	655	550	490	280	360	320	310
FIGlu conc.	0.094	0.013	0.060	0.040	0.046	0.063	0.062
Total FIGlu	61.57	71.50	29.40	11.20	16.56	20.16	19.72

Anaesthetist 3

Day	1	2	3	4	5	6	7
Urine volume	565	650	505	1440	680	670	570
FIGlu conc.	0.041	0.031	0.006	0.014	0.027	0.025	0.028
Total FIGlu	23.17	20.15	3.03	20.16	18.36	16.75	15.96

Anaesthetist 4

Day	1	2	3	4	5	6	7
Urine volume	660	680	710	630	670	420	590
FIGlu conc.	0.041	0.031	0.006	0.014	0.027	0.025	0.028
Total FIGlu	27.06	21.08	4.26	8.82	18.09	10.50	16.52

Anaesthetist 5

Day	1	2	3	4	5	6	7
Urine volume	1710	720	1040	800	970	1130	1170
FIGlu conc.	0.015	0.025	0.017	0.001	0.001	0.009	0.015
Total FIGlu	25.65	18.00	17.68	0.80	0.97	8.14	17.55

Table XVIII(cont)

Anaesthetist 6

Day	1	2	3	4	5	6	7
Urine volume	420	650	380	280	140	280	310
FIGlu conc.	0.053	0.037	0.064	0.071	0.070	0.052	0.047
Total FIGlu	22.26	24.05	24.32	19.88	10.64	14.56	14.57

Anaesthetist 7

Day	1	2	3	4	5	6	7
Urine volume	340	190	290	300	270	310	430
FIGlu conc.	0.011	0.044	0.034	0.042	0.040	0.039	0.018
Total FIGlu	3.74	8.36	9.86	12.60	10.80	12.09	7.74

Anaesthetist 8

Day	1	2	3	4	5	6	7
Urine volume	280	240	170	980	1050	550	720
FIGlu conc.	0.061	0.052	0.011	0.038	0.009	0.023	0.028
Total FIGlu	17.08	12.48	1.87	37.24	9.45	12.65	20.16

Anaesthetist 9

Day	1	2	3	4	5	6	7
Urine volume	630	460	740	760	530	590	30
FIGlu conc.	0.017	0.020	0.017	0.022	0.025	0.053	0.021
Total FIGlu	10.71	9.20	12.58	16.72	13.25	25.37	0.63

Anaesthetist 10

Day	1	2	3	4	5	6	7
Urine volume	630	615	950	520	440	640	530
FIGlu conc.	0.027	0.021	0.034	0.027	0.021	0.027	0.017
Total FIGlu	17.01	13.96	32.30	14.04	9.24	17.28	9.01

Table XIX. Time weighted mean (SD) and range of exposure to N_2O (ppm) for each anaesthetist.

Anaesthetist	Mean	SD	Range
1	135.6	112.3	47 - 325
2	115.2	85.7	22 - 229
3	119.8	134.0	32 - 356
4	60.8	66.5	0 - 139
5	94.8	77.4	0 - 193
6	85.2	86.1	14 - 226
7	159.2	184.3	0 - 413
8	53.4	45.5	0 - 107
9	109.8	166.7	12 - 407
10	58.2	47.4	0 - 114

CHAPTER SEVEN

DISCUSSION

Summary

This series of experiments has examined the effect of acute and chronic N_2O exposure on human folate metabolism using the formiminoglutamic acid (FIGlu) excretion test. Overall, it appears that acute N_2O exposure can cause a dose related increase in FIGlu excretion for 2 days. Brief exposure (<90 minutes) resulted in no increase whilst intermediate exposure (91-210 minutes) caused an increase in 50% of subjects. Prolonged exposure (>211 minutes) resulted in all subjects having an increase. Subjects with cancer had similar results. In contrast, anaesthetists exposed chronically to trace amounts of N_2O showed no effect. From these findings, it has been possible to show that folate metabolism becomes abnormal in people exposed to high concentrations of N_2O sooner than was previously thought. Conversely, and reassuringly, theatre pollution by N_2O appears to have little effect on the FIGlu excretion of anaesthetists.

Discussion

The FIGlu excretion test was used to assess folate metabolism for two reasons. Firstly, it is a non-invasive test requiring only histidine ingestion and urine collection. Secondly, this test has already been validated as being a good method of assessing cellular folate metabolism. Cellular folate metabolism was examined because it is the link between methionine synthase (MS),

the enzyme inhibited by N_2O, and DNA synthesis.

How valid is this test when the subjects are undergoing surgery? Can the results be extrapolated to assess N_2O toxicity? FIGlu excretion depends on several factors including the histidine dose, its absorption into the body, the volume of urine produced and the time it is collected for, the amount of cellular tetrahydrofolic acid (THF) present and the quantity of histidine catabolised to FIGlu.

Most previous studies have used 15 g oral histidine loading doses while in this series of experiments, 10 g was used. This dosage was chosen for several reasons. Firstly, histidine monochloride is expensive and difficult to obtain and therefore a lower dose was more economical. Secondly, it has been shown that any loading dose from 10 - 20 g is equally effective in diagnosing marginal folate/vitamin B_{12} abnormalities (Luhby and others 1958, 1959). Thirdly, the larger the dose, the greater the incidence of nausea and vomiting. This is the most common post-operative complaint of anaesthesia (Cohen and others 1986) and increasing its incidence by giving large histidine doses is both unacceptable to the patient and will reduce the total amount absorbed. Even with this low dose, 9 patients still had severe nausea and withdrew from the study. Fourthly, there was the possibility that large doses would result in accumulation of histidine overwhelming its catabolic pathway. No cumulative effect on urinary FIGlu concentration by continuous daily administration of 10 g histidine in the control subjects

was seen. However, it is possible that the 10 g dose may have allowed subjects with borderline abnormal folate metabolism to remain undetected which will have reduced the sensitivity of the test although its specificity should be maintained.

Absorption of histidine from the gastrointestinal tract may have been decreased as surgery inhibits proper gut function. However, histidine was given in a small volume at least 2 hours before the operation and most should have been absorbed. The operations were peripheral, and disturbance of gut function would have been minimal. All subjects were eating and drinking on the first post-operative day indicating that all histidine given after this was absorbed. The subjects undergoing thoracotomy or laparotomy are more likely to have suffered from decreased absorption. Whilst histidine was placed down a nasogastric tube, aspiration produced only small amounts of gastric fluid and so it appears that most of the histidine was absorbed. If absorption was reduced, the overall effect would have been to decrease the effective loading dose and therefore reduce the sensitivity of the test.

Urine output for all the subjects was maintained as customary for these operations and none became oliguric. Collection was over 8 hours. Many previous studies have measured urocanic acid excretion in the belief that it increases the test's sensitivity. This compound is an intermediate in FIGlu production.

$$\text{Histidine} \xrightarrow{\text{Urocanase}} \text{Urocanic acid} \longrightarrow \text{FIGlu}$$

It is often excreted together with FIGlu, occasionally in large amounts and has been measured both alone and together with FIGlu as a total histidine derivative. Chanarin (1963) showed it to be present in 88% of urine samples when 15 g oral histidine was given and in 42% of these it was the main histidine derivative. Davis and Kelly (1964) assayed 25 control subjects (loading dose 15 g oral histidine) and 8 excreted urocanic acid. Out of 31 patients with a vitamin B_{12} deficiency, 21 and 25 had raised FIGlu and urocanic acid excretions respectively. However, urocanic acid excretion is associated with many different conditions (e.g. liver disease, protein malnutrition, thyrotoxicosis, certain malignancies and TB) and this makes it a less specific test for folate/vitamin B_{12} abnormalities (Todays Tests 1969). The assay system used in these studies specifically measures FIGlu and causes no catabolism of urocanic acid. It is possible that some of the subjects had raised urocanic acid, but normal FIGlu excretions. However, as other reasons may have caused this, it was decided to use the more specific assay.

Therefore the FIGlu excretion test was designed to specifically measure urinary FIGlu and to use a histidine loading dose that was acceptable to the patient whilst being large enough to diagnose abnormalities. It is unlikely that the operations had much effect on either absorption of histidine or excretion of FIGlu. It is possible, however, that a few subjects with an abnormal

folate metabolism were not detected and the test may have a reduced sensitivity in these circumstances.

The FIGlu excretion of the control subjects varied widely (range 0.33 to 74.3 μmol). It is difficult to compare this to other studies as many different assay systems, loading doses and urine collection times have been used although the results of this study are in approximate agreement with many of the other studies (Table XX).

	Histidine loading dose g	Collection Time hours	FIGlu Urine excretion μmol Range	Mean
Carey et al 1964 Electrophoresis	20	4	0-110	40
Davis et al 1963 Enzymatic	15	8	0-95	44
Luhby et al 1959 Enzymatic	15	24	0-29	3.5
Chanarin et al 1962 Enzymatic	15	8	5-98	52

Table XX. FIGlu excretion determined in other studies in normal groups.

The mean age of the control group is significantly lower than those of the other groups. On group who used no loading dose and an enzymatic assay found that there was a slight increase in FIGlu excretion in the 40-50 year olds as compared to other ages (Rosenauerova-Ostra and others

1976). Koblin and Tomerson (1990) found that age had an]effect with older rats excreting more FIGlu after N_2O exposure. However, no loading dose was used and it is possible that older rats have a different metabolic rate in respect to protein and histidine catabolism. In addition, vitamin B_{12} concentrations were not measured and it is possible that they are reduced in the older rat. The control group was comparable to the other groups in all other aspects. All subjects acted as their own controls on the pre-operative day and these were similar to the 10 control subjects although those patients with tumours had a non significant increase in excretion.

None of the subjects undergoing surgery showed any evidence of marrow depression in their blood films. This was expected as any changes are indicative of severe and prolonged marrow depression. It thus appears that exposure to N_2O for up to 6 hours has no gross effect on bone marrow. FIGlu excretion was increased on the first and second post-operative days for group 2 subjects indicating either a cellular deficiency of THF or a surgical effect although group 3 subjects were unaffected. One possible explanation could be that the raised excretion was due to the stress response of surgery. Central nerve blockade prevents this during surgery of the lower limb (Pflug and Halter 1981, Stefansson and others 1982). However, blockade was brief, lasting only a couple of hours and the stress response would occur after this in both epidural (Gordon and others 1973) and spinal anaesthesia (Moller and others 1985). It would be expected that nerve blockade

would be unlikely to prevent the increase in FIGlu excretion on the next 2 days.

The other possibility is that it could be related to the general anaesthetic drugs, thiopentone or enflurane, although they have not been implicated in folate metabolism previously. Therefore, it appears that N_2O exposure causes an abnormality in the functional THF pathway in 50% of subjects exposed to N_2O for more than 90 minutes and this lasts for up to 48 hours. This agrees with the time taken for MS recovery (Deacon and others 1980, Kondo and others 1981, Koblin and others 1981).

Of interest is the increase in excretion that occurred in patients exposed to N_2O for only a relatively short time. Work has shown that the inhibitory $t_{\frac{1}{2}}$ for MS by 70% N_2O is 46 minutes (95% CI 30-99 minutes) (Royston and others 1988) and this would indicate that approximately 2-8% of MS would still be active after 159 N_2O minutes exposure. However, some increases were seen in patients with shorter exposure times (down to 90 minutes). How is it possible that these individuals had a raised excretion as theoretically there would still be 25% MS activity present?

A second interesting observation is the finding that whilst those subjects exposed to N_2O for a long time (>211 minutes) all excreted raised FIGlu and those exposed for a brief time (<90 minutes) had no increase, the subjects who had an intermediate exposure had a 50% probability of raised excretion which was not related to duration. Hawkins and others (1987) showed an increase in excretion

in 2/6 subjects who were exposed to hyperbaric N_2O. Even though it is difficult to compare these findings, it is in agreement with the present studies. Why should some subjects be more susceptible to the effects of N_2O?

Various possibilities may explain these apparent anomalies. Firstly, MS activity shows great individual variation and there may exist people with intrinsically low MS activity. Such individuals may be especially vulnerable to N_2O as only a small amount of MS inhibition would cause an overall significant decrease in activity.

Secondly, the amount of MS that has to inhibited before clinical effects occur is unknown. Presumably, less inhibition is needed to cause folate abnormalities than for clinical effects. It is possible that there is great individual variation in the relationship between the amount of MS inhibited and folate status. Some people who are resistant to N_2O may only develop abnormal folate metabolism if most of their MS is inhibited whilst others who are less resistant will show changes in metabolism when small amounts of MS are inactivated. Presumably these individuals will also demonstrate more obvious clinical effects at an a earlier stage. Why this should be is unknown.

Thirdly, it may be that individuals have different susceptibilities to MS inactivation with some being more sensitive and others more resistant. This may be related to the intrinsic amount of MS present, but also may be related to how MS is inhibited. If, as suggested, it is due to formation of a hydroxyl radical (Koblin and

Tomerson 1990), then cells may have a protective mechanism whose efficiency could vary greatly. This would explain the interspecies difference in MS inhibition as different animals may possess different amounts of the scavenging system.

If this mechanism exists, 2 possibilities could be considered. Firstly, there may be a group of humans who are especially sensitive to MS inactivation and who will demonstrate clinical effects more readily. Ill patients may be more prone to the toxic effects of N_2O (Amos and others 1982) and it could be possible that gross physical illness may, in some way, interfere with the scavenging system. If individuals have other abnormalities causing increased sensitivity to N_2O (decreased folate, vitamin B_{12}, etc), then they may be especially sensitive. One possibilty for this apparent difference in susceptibilities could be because glutathione (GSH) is the scavenging system. It can scavenge hydroxyl radicals and is present in high concentrations in cells and is readily oxidised.

$$2GSH \xrightarrow{OH^{\cdot}} GSSG + NADPH + H^{+} \longrightarrow 2GSH + NADP^{+}$$

The oxidised glutathione (GSSG) is reduced back to GSH by glutathionine reductase. Normally there is a high GSH:GSSG ratio in the cells as oxidised GSSG can inhibit sulphur containing enzymes;

$$\text{Enzyme-SH} + GSSG \longrightarrow \text{Enzyme-S-SG} + GSH$$

However, it is possible that with large

concentrations of hydroxyl radicals, the rate that GSSG is reduced back to GSH is too slow to prevent a decrease in GSH:GSSG resulting in damage to the active site of MS by both GSSG and OH$^{\bullet}$. If the cellular concentrations of GSH are low, then MS inhibition may occur sooner. Alternative scavenging systems include uric acid (Ames 1981).

The second possibility is that there may be a better method of protecting a patient from N_2O toxicity than using folinic acid. After MS is inhibited, folinic acid acts as an escape mechanism for DNA synthesis. However, it is better if the hydroxyl radical is scavenged before MS is inhibited. This method has been used successfully in mice given dimethylthiourea (Koblin and Tomerson 1990). Other, less toxic, compounds exist that can scavenge hydroxyl radicals (Halliwell and Gutteridge 1985). For example, vitamin C reacts rapidly with OH$^{\bullet}$

Ascorbate + OH$^{\bullet}$ -----> Semihydroascorbate

2Semihydroascorbate -----> Ascorbate + dehydroascorbate

The dehydroascorbate breaks down in a complicated fashion to form oxalic and threonic acids. Therefore high cellular concentrations of vitamin C may protect MS. Many other compounds can react with the hydroxyl radical (e.g. glucose, bicarbonate ,etc - Anbar and Neta 1967) and these could also be used. This remains to be investigated.

It was interesting to note that one patient who had a

low vitamin B_{12} concentration excreted normal amounts of FIGlu. It has been suggested that these individuals are more prone to N_2O effects although there is little evidence for this in man. Unfortunately other possible groups that may be more susceptible were not studied.

One group that is likely to have abnormal folate metabolism was studied. Tumour cells have an increased requirement for folate in a similar manner to that of rapidly proliferating tissue (Rama Rao 1964). FIGlu excretion in subjects with cancer was very similar to that seen in non-cancerous subjects. Pre-operative excretion was slightly raised as compared to normal controls. Interestingly, 3 of the patients undergoing lung resection received only a small amount of 70% N_2O (12, 18 and 24 minutes) and all these had normal FIGlu excretion, further evidence that the stress of surgery is not responsible for raising FIGlu excretion. Excretion in the other subjects was similar to that in group 2 patients with approximately 50% showing a raised excretion. The duration of 70% N_2O exposure was 96 (SD 32) minutes, less than of the non-tumourous patients indicating that even though the increased excretion was similar, exposure was less. Possibily these patients are more susceptible, but this needs further study.

Patients undergoing oesophagectomy for oesophageal cancer are often malnourished with a vitamin deficiency although there was no evidence of this in these patients. FIGlu excretion was similar to the lung tumour group, although N_2O exposure was longer (mean 154 (SD 42)

minutes).

It appears therefore that patients with cancer may have an increase in FIGlu excretion when compared to normal patients undergoing N_2O anaesthesia and therefore it is possible that cancer may increase the effects of N_2O on folate metabolism. Extrapolation from this result to other groups with rapidly growing tissues (pregnant women, young children, etc) whilst possible, must be done with care. However, the effect appears at worst to be minor.

Daily urinary excretion of FIGlu by anaesthetists exposed to N_2O pollution is similar to that of normal unexposed subjects, indicating that the chronic exposure to N_2O of anaesthetists had no effect on folate metabolism and this is in agreement with other studies (Salo and others 1984, Nunn and others 1982). The amount of N_2O pollution varied widely and was greatly reduced by scavenging. Most anaesthetists work in scavenged theatres and had exposures to N_2O that are consistent with these other studies (Davenport and others 1980, Gray 1989).

This result, taken in context with the other biochemical studies and with the epidemiological evidence is reassuring for anaesthetists and other theatre workers. Chronic exposure to low concentrations of N_2O appears not to be a health hazard except for a possible detrimental effect on fetal development. However, there is still a lack of evidence about the safety of N_2O when worse pollution occurs (e.g. dental surgeries) and it is possible that the effect may become toxic. At what concentration of N_2O contamination danger occurs is

unknown. The target exposure concentration set by the United States National Institute for Occupational Safety and Health is 25 ppm. These targets were almost always superseded in this study and it appears that this concentration may be unnecessarily low. A greater higher exposure concentration appears to be not only safe, but also more realistically obtainable.

Increase in FIGlu excretion due to abnormal folate metabolism is an indicator that the effects of N_2O are more profound at a lower exposure dose than have been previously reported. However, it is still impossible from these observations to make deductions about the direct clinical effects of N_2O.

In conclusion, several possible conclusions may be made from these results.

1. This test may be used to investigate N_2O toxicity by examination of folate metabolism. The test will tend to underestimate abnormalities.

2. It appears that people may be more susceptible to N_2O than has been previously thought. Folate metabolism is affected in a dose dependent manner. Some individuals appear to be more susceptible than others and possible reasons are discussed. Whether the derangement in folate metabolism is of clinical significance is still unknown

3. Tumour patients may be more susceptible to N_2O, with effects occurring at lower concentrations. This may have relevance to other groups with rapidly growing

tissues.

4. Occupational exposure to the N_2O pollution concentrations studied (approximately 100 ppm) have no effect on folate metabolism.

CHAPTER EIGHT

NITROUS OXIDE IN MODERN ANAESTHESIA

A PERSONAL VIEW

Both acute and chronic nitrous oxide (N_2O) exposure interfere with vitamin B_{12} and, consequently can result in a reduction in DNA synthesis. Chronic exposure can cause a neurological disease with similar symptoms to those of vitamin B_{12} deficiency. These are biochemical toxic effects unique to N_2O. However, N_2O causes other complications which influence morbidity and mortality after surgery (Table XX). Nunn has suggested that N_2O would not receive a product certificate if it was introduced into anaesthesia as a new drug because of these problems (Nunn 1987). Some anaesthetists have even suggested banning its use altogether in anaesthesia because of these side effects (Eger 1985).

However, this solution is drastic because there are undoubted advantages in its use. It is a popular drug having been used since 1844 in countless millions of anaesthetics. For example an estimated 1×10^9 litres were used in 1988 by British anaesthetists alone (Editorial 1989). If it was banned, most anaesthetists would have to make significant modifications to their practice.

General anaesthesia is considered to comprise of 3 components, narcosis, reflex suppression (analgesia) and muscle relaxation. Anaesthesia involves one or all of these and anaesthetic drugs vary in which they affect. N_2O can cause narcosis and analgesia, but no muscle relaxation and so this area will not be discussed further. If N_2O was banned, techniques which give an equivalent amount of narcosis and reflex suppression would be need to be developed. Three methods are available, to use more

Table XX. Effects of nitrous oxide

Advantages	Disadvantages
Non-flammable/explosive	Inhibits MS Marrow depression Reproductive effect ? immune system ? wound healing
Amnesic	Expensive, storage
Analgesic	Patient delivery problems Expensive equipment
Fast Induction Low blood/gas solubility Non-irritant Odourless Concentration Effect 2nd gas effect	Hypoxic gas mixtures Contamination Atmosphere pollution
Fast recovery	Weak anaesthetic Awareness Light anaesthesia
Decreases other anaesthetic use	Diffusion potential Non-compliant space Compliant spaces Diffusion hypoxia
Little cardiorespiratory effect ? ischaemia ? arrhythmias	Decreases oxygen supply Central nervous system Acute Chronic

Possible Disadvantages

Emetic
Slow bowel recovery
Malignant hypertension
Liver damage
Renal damage
Delayed lung recovery

volatile anaesthetics, to use intravenous techniques or to use local anaesthesia.

Whilst local anaesthesia is common, it has several disadvantages including a slow onset time which can delay surgery, a greater failure rate than general anaesthesia, and is disliked by some patients. Anaesthetists often use local anaesthesia alone or as part of a "balanced anaesthetic technique". However, regional anaesthesia will not be discussed further because it is not universally applicable to all operations.

To clinically assess an anaesthetic drug, 2 factors are important. Firstly, how effective an anaesthetic is it? Because surgical stimuli vary greatly, so must anaesthetic depth. Inadequate narcosis or reflex suppression (light anaesthesia) may cause awareness and recall, operating conditions will be poor and potentially dangerous physiological responses can occur (hypertension, tachycardia, etc). In contrast, with deep anaesthesia, recovery may be prolonged and dangerous cardiorespiratory depression may occur. An anaesthetic drug (or combination) should be able to anaesthetise patients to a suitable depth and then be able to vary this quickly.

The second factor concerns the incidence of side effects that a drug has. A good drug has only a few minor ones. Therefore the most suitable anaesthetic drug (or technique) is one that gives effective anaesthesia with few side effects. An ideal anaesthetic drug should have many characteristics (Editorial 1990). It should be easily and inexpensively prepared in its pure form, stable

without preservatives with a long shelf life, be non-flammable or explosive, potent to allow high concentrations of oxygen to be used, possess a low blood/gas partition coefficient to allow a fast induction and emergence and flexibility in adjusting anaesthetic depth, pleasant to inhale, have no effects on the cardiovascular and respiratory systems, should depress specific areas of the central nervous system causing anaesthesia, but no stimulation, undergo no bio-transformation and lastly, have no organ toxicity.

Assessment of the role N_2O has in anaesthesia must consider these 2 areas. In this chapter, the advantages and disadvantages of N_2O will first be discussed and then they will be compared to those of the above techniques.

The first reported death from N_2O inhalation was published in 1873. Miss Ida Wyndham died during a N_2O anaesthetic for the unsuccessful removal of an upper molar tooth, probably due to asphyxia (Lancet 1873). Since then, there have been many other fatalities from the use of N_2O, some of which will be detailed later in this chapter.

Nitrous oxide is manufactured by heating ammonium nitrate (Wynne 1985).

$$NH4NO3 \text{ ------> } N_2O + 2H2O + 58.6kJ$$

Older manufacturing plants use solid ammonium nitrate heated to 245° - 270°C whilst modern ones use 83% aqueous ammonium nitrate which is cheaply obtained as a byproduct of ammonia manufacture. The reaction is exothermic and

requires strict control to prevent the temperature rising because ammonium nitrate explodes when too hot. There have been 25 major explosions related to N_2O manufacture which have injured or killed over 5,000 people in the last 100 years (Wynne 1985). High temperatures also increase N_2O contamination by the higher oxides of nitrogen. Other impurities may be present including carbon monoxide, chlorine and ammonia. These are formed as a result of side reactions occurring during manufacture.

$$5NH_4NO_3 \text{ -----> } 4N_2 + 9H_2O + 2NHO_3 + 157.0kJ$$

$$2NH_4NO_3 \text{ -----> } 2NO + N_2 + 4H_2O + 54.4\ kJ$$

$$2NH_4NO_3 \text{ -----> } 2N_2 + O_2 + 4H_2O + 146.5kJ$$

$$\text{Then} \quad 2NO + O_2 \text{ -----> } 2NO_2$$

These impurities are removed by passing the gas through water, caustic soda, sulphuric acid and alkaline potassium permanganate. However, problems have occurred in the purification process. In 1966 there were 2 deaths from N_2O contamination by nitric oxide. Met-haemaglobinaemia, pulmonary oedema and circulatory collapse occurred (Clutton-Brook 1966) and investigations revealed that N_2O contamination was relatively common and probably had been responsible for a considerable mortality in the past (Editorial 1967). Modern regulations allow N_2O to be contaminated by up to 1 ppm nitrogen dioxide, 1 ppm halogens, 10 ppm carbon monoxide, 300 ppm carbon dioxide and 25 ppm ammonia (U.S. Pharmacopoeia). Stringent manufacturing safeguards and checks make it unlikely that dangerous contamination can now occur although this

potential will always exist.

N_2O delivery to the patient can cause problems with too much, too little, or the wrong gas being inhaled. N_2O is supplied to theatres either in cylinders or piped from a central depot. Cylinder use has obvious problems because they are cumbersome and dirty and can empty quickly causing the patient's anaesthetic depth to lighten, or if the fresh gas flow decreases, can cause rebreathing with CO2 accumulation. Even though modern cylinders use a pin index system, wrong ones may still be fixed to the N_2O port.

Equally, there are problems in using piped N_2O. Different pipes carrying other gases may be abnormally connected with the wrong gas being delivered to the theatre. A new emergency treatment room with outpatient operating theatre facilities was built in Canada. After having been used for 5 months, a young girl died from asphyxia during an operation and the cause was found to be a misconnection of the oxygen and N_2O pipelines during installation. A subsequent inquiry suggested that this problem had been involved in the deaths of another 22 patients (Pelton 1978).

Whilst safeguards have been developed preventing this, whenever a hypoxic gas is connected to an anaesthetic machine it may be accidently given instead of oxygen with disastrous consequences. Modern safeguards (coloured cylinders, pipelines, pin index system, oxygen analysers, etc) have reduced the possibility, although mistakes can always be made. For example there have been

several documented cases of cylinders being attached to the wrong pin index (Goebel 1980). Rotameters may be accidently set to give a hypoxic gas mixture although modern anaesthetic machines are designed to prevent this (Quantiflex rotameters, etc). However, the only foolproof method of preventing the wrong gas being given is not to have any hypoxic gas (N_2O and carbon dioxide) on the anaesthetic machine.

Once formed, it is a stable gas with a long shelf life and an atmospheric lifetime of about 150 years (Editorial 1990). It is expensive. For example, in 1975, it cost as much to use as halothane (Barton and Nunn 1975). Less is needed with closed circuit anaesthesia although this technique again requires expensive equipment and exposes patients to new hazards.

It is non-flammable or explosive, but if heated will decompose into nitrogen and oxygen (450°C) and this mixture strongly enhances combustion of other compounds (Mushin and Jones 1984).

There is concern that environmental pollution from industrial wastes can affect the ozone layer or could increase the "greenhouse" effect. N_2O has been implicated because of its long atmospheric life. It is a significant "greenhouse" gas and its atmospheric concentration is increasing. From 1979 to 1982 there was an annual increase of ≈0.8 ppb (0.25% yearly)(Khalil and Rasmussen). N_2O has a similar molecular potency to CO_2 in causing the "greenhouse effect". However, many other N_2O sources exist including microbial nitrification and denitrification of

fertilisers and anaesthesia's contribution (estimated at 1 x 10^9 litres in 1988) is small when compared to these sources (3-10% of microbial nitrification alone - Logan 1989).

N_2O has no direct ozone effect although it can be oxidised to nitric oxide which then enters the NO_x catalytic cycle. This works in the same way as chlorine in causing ozone depletion. The oxides of nitrogen are only removed when they react with other radicals and hence can inflict great damage on the ozone layer (Brown and others 1989).

The potential health hazard of exposure to trace concentrations of N_2O from theatre pollution has been previously discussed. In addition, it may also cause behavioural problems although most studies show no or only a small effect. Cook and others (1978) exposed 29 healthy males to up to 0.4% N_2O and found no change in the digit span test (memory) or the choice reaction time (attention). Halothane (20 ppm) and N_2O (500 ppm) combinations had no effect. Whilst sub-anaesthetic concentrations have a behavioural effect, 20-30% N_2O is needed (Rice 1983). Twenty two anaesthetists (time averaged exposure to N2O was 58 ppm and to halothane was 1.4 ppm) had normal mood and cognitive test results after working in theatre (Stollery and others 1988). Therefore, at normal theatre concentrations, N_2O appears to have little behavioural effect.

It is a weak anaesthetic having a high minimum alveolar concentration (MAC) of 104% (SE $\pm$ 10%). To find

its MAC, volunteers were anaesthetised with only N_2O in a pressure chamber and movement to an electrical stimulus assessed (Hornbein and others 1982). In normal anaesthesia, lower concentrations are given and therefore other anaesthetics are needed to prevent light anaesthesia. If given alone (usually in concentrations of 50 - 70%), the patient will be poorly anaesthetised and awareness may occur. For amnesia, 65-86% nitrous oxide is required. When 42 patients were given 70% N_2O and curare, 3 were aware (Taub and Eisenberg 1975). Inhaling only 70% N_2O will always cause some awareness.

In contrast to its weak anaesthetic actions, it is a powerful analgesic with 20% N_2O being as effective as 15 mg subcutaneous morphine (Chapman and others 1943). Analgesia is dose related (Dundee and others 1962) and may be partially due to interaction with the endorphin receptor system. N_2O decreased the pain of a tight tourniquet and intravenous 4-8 mg naloxone partially reversed this (Yang and other 1980). Other studies have shown similar results although high doses of naloxone are required suggesting that the N_2O-endorphin receptor interaction is complicated and other effects may be important (Finck 1985).

Its analgesic effects are used in childbirth and relief of acute pain. Portable cylinders are readily available which contain Entonox, a mixture of oxygen and nitrous oxide (50:50). Analgesia is not instantaneous and Entonox must be inhaled for some time. For instance, it takes 45 seconds before analgesia becomes satisfactory in

the first stage of labour. Forty six percent of mothers had effective analgesia when using it although another 30% had no relief (Holdcroft and Morgan 1974). Other uses for Entonox include pain relief post-operatively (Parbrook 1967), for painful minor procedures (Baskett and Bennett 1971) and in ambulances (Stewart 1983). Storing Entonox is a problem because it is normally a gas mixture (Poynting Effect), but when cooled below -7°C, the N_2O liquefies. If it is now used, only oxygen will be inhaled giving no analgesia. As the oxygen is used up, further inhalation will result in an increase in N_2O intake and hypoxia may occur. Therefore, it needs to be stored in areas where the temperature does not fall. If liquefication occurs, leaving it at room temperature for 2 hours reverses the changes.

When used at anaesthetic concentrations, it has few effects on either the cardiovascular or the respiratory systems although there is concern over possible ischaemic and arrhythmogenic effects. All anaesthetic drugs decrease tidal volume and increase respiratory rate and N_2O is no exception, although the respiratory rate increases enough to prevent decreases in minute alveolar ventilation (at 1.55 atmospheres (atm), the respiratory rate was 47.4 ± 2.5 breaths.minute^{-1} (Hornbein and others 1982)). It reduces the ventilatory response to hypercapnia and hypoxia and at 0.1 MAC these decreased by 45% and 42% respectively. This is similar to the other anaesthetics (Knill and Clement 1982).

N_2O can cause a tight chest and may make assisted

ventilation more difficult. When 70% N_2O and morphine (2 $mg.kg^{-1}$) were given, there was an increase in the rigidity of the abdominal wall and a decrease in chest compliance (Freund and others 1973). Whilst morphine may have been responsible, a similar rigidity was seen when only 1.55 atm N_2O was inhaled (Hornbein and others 1982).

N_2O may increase the incidence of post-operative chest complications. Gawley and Dundee (1981) studied 85 patients undergoing non-emergency upper abdominal operations anaesthetised with either 66% N_2O and a volatile anaesthetic or with a volatile anaesthetic alone (but the gases were humidified). The N_2O group had more respiratory complications and lower arterial oxygen saturations. This, however, may have been due to humidification of the gases. Another study compared post-operative sequelae in upper abdominal operations in 48 patients anaesthetised with either 30% oxygen in nitrogen or N_2O and showed no difference (Logan and others 1977). Lampe and others (1990) studied 270 patients receiving hip replacements. They used an isoflurane-vecuronium anaesthesia with either 100% O2 or 60% N_2O. Ventilation was controlled and the gases humidified. Fentanyl was given as needed with both groups receiving similar doses. Pulse oximetry measured arterial oxygen saturation pre-operatively, intra-operatively, 60, 120 minutes post-operatively and on days 1, 2, 3 and 5 after 5 minutes of breathing room air. Intra-operative saturation was lower in the N_2O group (93.3%$\pm$2.8% v 96.0%$\pm$5.2%). Immediate and later post-operative saturations were the same for both

groups. Similar findings occurred on days 1 to 5. Because oxygen can cause absorption collapse, nitrogen may have been a more suitable control gas. However, from these studies, it appears that N_2O has little deleterious post-operative effects although a larger study is still needed. In addition, there may be a subgroup of patients with existing respiratory abnormalities who may be more susceptible.

It has little effect on the cardiovascular system when compared to the volatile anaesthetics. It depresses cardiac muscle contractility slightly. Eighty percent N_2O reduced it in dog myocardial perfusates by 25% (Motomura and others 1984). Because it causes sympathetic stimulation, this depression is usually of no clinical relevance because blood pressure is usually maintained (Eisele and Smith 1972). Its cardiovascular stability gives N_2O a great advantage over the other volatile anaesthetics which all depress either the myocardium or the peripheral circulation. However, patients who already are maximally sympathetically stimulated may become hypotensive when given N_2O (e.g. shocked patients).

Pulmonary artery pressure and vascular resistance are not normally changed unless pulmonary hypertension is already present when large increases in resistance and pressure can occur (Schulte-Sasse and others 1982). However, in another study when 70% N_2O was given to 10 patients with pulmonary hypertension, there was a decrease in pulmonary arterial pressure suggesting that pulmonary vascular resistance had not increased (Konstadt and others

1990). More work is needed on this subject.

There has been concern that N_2O may adversely affect patients with ischaemic heart disease. Philbin and others (1985) used a canine model with a critical stenosis of the left anterior descending coronary artery. Increased dysfunction of the supplied myocardium occurred when 60% N_2O was given. Other work has shown similar cardiac dysfunctions at N_2O concentrations of 40% although the effect was small (Leone and others 1988). The clinical relevance of this is still unknown. Patients undergoing carotid endarterectomy (high incidence of coronary artery disease) were given isoflurane-fentanyl-vecuronium with either 100% O2 or 60% N_2O in O2 and the incidence of cardiac ischaemia was found using 12 lead ECG and trans-oesophageal echocardiography (TEE). Seventy patients were studied, randomly allocated to either group; both had a similar degree of previous myocardial infarction (20%) and ischaemia (10%). Intra-operative cardiovascular drug usage was similar. Twenty one patients in both groups had one or more haemodynamic events (30% change in baseline systolic pressure) and there was no difference in intra-operative ischaemia or TEE abnormalities. Other studies have found similar clinical findings. Slavik and others (1988) studied 7 patients undergoing bypass for coronary artery disease using TEE and gave, in 10 minute sequences, 100% O2, 70% N_2O in O2, 70% N2 in O2 and 100% O2. No abnormal wall motion was seen. Mitchell and others (1989) examined 70 patients undergoing coronary artery grafting using a crossover design with either 60% N_2O or N2 in O2.

N_2O had no effect on TEE determined ischaemia. Therefore it appears that there is little clinical effect although larger studies are needed.

N_2O may possibly be arrhythmogenic because it can activate the sympathetic nervous system and increase plasma catecholamine concentrations (Roizen and others 1987). One hundred patients undergoing trans-sphenoidal hypophysectomy received either 100% O2 or 60% N_2O in O2. Adrenaline, 1:200,000 in 0.5% lignocaine was given (mean dose 2.6 $\mu g.kg^{-1}$) and the ECG was monitored for 15 minutes. N_2O increased the number of premature ventricular beats although this was not statistically significant. In addition, isorhythmic AV dissociation was increased by 50%. It appears therefore that N_2O does increase arrhythmias - more work is needed to assess the clinical dangers (if any) of this.

In conclusion, N_2O has little effect on the respiratory system apart from a possible increase in post-operative complications. It has a small cardio-depressant effect, but because it causes sympathetic stimulation, blood pressure is maintained. Arrhythmias may be a problem and its use in patients with myocardial ischaemia is still controversial.

Its blood/gas partition coefficient (0.46) is the lowest of all the common inhalational anaesthetics and therefore, it is the least soluble in blood. This, theoretically, will allow a rapid induction. Its alveolar concentration will rapidly approach that of the inspired air long before other anaesthetics because only a little

is removed by blood. The concentration effect also increases the speed of induction. Smith and others (1974) anaesthetised 2 volunteers with the same amount of N_2O (approximately 1 atm) at different pressures of 2 atm (50% N_2O) and 1.2 atm (85% N_2O). Induction was faster when 85% N_2O was used. The second gas effect is enhanced by N_2O (Epstein and others 1964). All these effects combined with its lack of odour and its non irritability to the respiratory tract makes N_2O an excellent induction drug.

Recovery is equally rapid because N_2O is quickly exhaled from the lungs. There is the possibility that hypoxia could develop during recovery because N_2O will dilute the other alveolar gases including oxygen (diffusion hypoxia). It however appears that this is only of minor clinical importance in most patients because it is mild and short lived (Frumin and Edelist 1969). If there is anxiety about hypoxia at this stage, oxygen prevents it.

N_2O readily diffuses into body spaces that contain air. Diffusion is related to the partial pressures of the gases between the different media (Fick's Law). When one of these media is a fluid then diffusion is related to the solubility of the gas in the liquid. The more soluble the gas, the greater its diffusing capability. N_2O is more soluble in blood than nitrogen (blood/gas coefficient 0.015) by a factor of 25. Gas in body spaces is in equilibria with that dissolved in blood and therefore normally, spaces will contain nitrogen, oxygen and carbon

dioxide. If N_2O is inhaled in oxygen with no rebreathing, nitrogen will be exhaled and eventually all will be removed from the lungs, blood, tissues and body spaces and replaced with N_2O. Because more N_2O is in solution, it will diffuse faster into the body spaces than nitrogen can diffuse out and so there will be an increase in the amount of gas present. If N_2O continues to be inhaled, eventually all nitrogen will be removed and the total amount of gas present in the spaces will be the same as before. The converse will happen when N_2O is withdrawn.

This increase in the amount of gas present in the space before equilibrium can have two consequences. If the space is compliant there will be an increase in its volume which is theoretically equal to (Eger and Saidman 1965);

$$\frac{Fi N_2O \text{ in alveolar}}{(1.0 - Fi N_2O)}$$

Therefore inhalation of 70% N_2O can cause an increase in volume of a compliant body space of up to 233% However, if the space is non-compliant, there will be an increase in its gas pressure. Increases in pressure are related to the partial pressure of N_2O in the alveolae and can have significant clinical effects.

Compliant spaces include the stomach and intestines, a pneumothorax, a pneumo-peritoneum, surgical emphysema and air emboli. Normally, the intestines contain only small quantities of air and so 70% N_2O would only have a minor effect on volume. However, during intestinal obstruction, the quantity increases greatly and in this

situation, N_2O expansion may make abdominal closure difficult or increase intra-abdominal pressure. Fortunately, the rate that the volume increases is slow because loops of intestines from a dog exposed to 70% N_2O took 100 minutes to double in size (Eger and Saidman 1965). N_2O appears to have no effect on post-operative motility or gasto-intestinal function (Giuffre and Gross 1986). However, recent work has different results (Scheinin and others 1990) because 40 patients undergoing colonic surgery were anaesthetised with either isoflurane and air or isoflurane and N_2O (70%). Subjects in air group had less gas in their small bowels and the operating conditions were superior. Bowel function returned earlier as measured by passage of flatus and faeces and overall, they had a shorter hospital stay. However, air was placed in the stomach at the start of the anaesthetic and this could have had a significant effect. Further investigation is needed to determine the importance of this result.

Lung spaces can expand and squash remaining intact tissue when N_2O is inhaled. Experimentally, in dogs breathing 70% N_2O, a pneumothorax doubled and then tripled in size in 10 and 30 minutes respectively (Eger and Saidman 1965). This change is quicker than that occurring in the gut and its possible consequences are more severe. Air embolism is common in both open heart and neurosurgery. Air emboli size increases with N_2O and may become life threatening or lodge in vital arteries (e.g. brain) causing strokes. Inhalation of 72 - 76% N_2O reduced the amount of IV gas required to kill rabbits by up to

340% (Munson and Merrick 1966). Pigs had increased cardiovascular problems after cardiac bypass when given N_2O probably due to an expansion of air emboli (Tuman and others 1987). Therefore, its use is contraindicated in operations were air emboli may occur.

The cuff on an endotracheal tube may expand with an increase in mucosal pressure thereby increasing the incidence of sore throat or pressure necrosis. This occurs in a time dependent manner although it is most profound over the first 60 minutes (Mehta 1981). The effect can be avoided by using a variety of techniques including expanding the cuff with water or N_2O, using a valve to allow excess gas to bleed off or to manually check the cuff pressure.

Non-compliant spaces include the middle ear and nasal sinuses, a pneumo-encephalos and the eye. Operations on the middle ear are particularly prone to the diffusive effects of N_2O (Perreault and others 1982). Bulging of the drum can be seen which may result in rupture, graft displacement, stapes displacement, haematotympanum and hearing loss. Withdrawing N_2O 30 minutes before graft placement prevents this happening.

It can diffuse into any air spaces in the cranium possibly resulting in harmful pressure increases (Saidman and Eger 1965). These air spaces may be as result of trauma, investigative procedures (ventriculography, pneumo-encephalography) or treatment (CSF shunt, posterior fossa surgery). When there is a possibility of a closed airspace in the cranium, N_2O should not be used.

Gas is often injected into the eye (usually sulphur hexaflouride) after surgery for retinal detachment in order to tamponade the retina and N_2O can cause a considerable increase in intraocular pressures and it is recommended that it be stopped 15 minutes before injection (Stinson and Donlon 1982). There appears to be little effect in surgical emphysema (Poulton and others 1982), but it is possible that increases in the size of the balloon in flotation catheters could distend the pulmonary artery although an in vivo study showed no effect (du Bouley and Nahrwold 1982).

Hence, N_2O may cause considerable morbidity or mortality as a result of its low solubility. The effects are predictable and in high risk cases N_2O should not be used. However, these spaces may be present without the anaesthetist being aware of them.

N_2O may cause nausea and vomiting for several reasons. It diffuses into the gut causing distension, into the middle ear causing an increased pressure, it can interact with the endogenous opioid receptor systems and it activates the sympathetic nervous system (Melnick and Johnston 1987). All 7 volunteers anaesthetised with N_2O at 1.55 atm had post-anaesthetic nausea and vomiting suggesting that it has emetic properties (Hornbein and others 1982). Many clinical trials examining this have been performed (Table XXI).

Alexander and others (1984) studied 74 women undergoing laparoscopy and showed an increase in the

incidence of nausea and vomiting in those receiving 70% N_2O and fentanyl as compared to patients who received

Table XXI. The Emetic potential of nitrous oxide.

Trial	Number	Operation	Groups	N + V
Alexander and others 1984	74	Laparoscopy	70% N_2O/fent	61%
			Iso	25%
			Iso/fent	30%
Lonie and Harper 1986	87	Laparoscopy	67% N_2O/enf/fent	49%
			Enf/fent	17%
Melnick and Johnston 1987	60	Minor gynae	60% N_2O/Iso	25%
			Iso	3%
Muir and others 1987	780	Non-abdominal surgery	Enf/67% N_2O	10%
			Enf	11%
			Iso/67% N_2O	12%
			Iso	9%
Kortilla and others 1987	110	Hysterectomy	Fent/Iso/70% N_2O	67%
			Fent/Iso	62%
Hovorka and others 1989	150	Laparoscopy	Enf/70% N_2O	54%
			Iso/70% N_2O	48%
			Iso	52%
Eger and others 1990	270	Variety	Iso/60% N_2O	46%
			Iso	34%

isoflurane alone or with fentanyl. Melnick and Johnston (1987) found that in 60 women undergoing minor gynaecological surgery, 60% N_2O with isoflurane caused an increase in the incidence of nausea and vomiting (25%). No narcotics were used and the operations did not involve

the gastrointestinal system. Lonie and Harper (1986) showed that the incidence of vomiting in 87 patients undergoing laparoscopy decreased from 49% to 17% when no N_2O was given.

However, other work has failed to demonstrate this. Muir and others (1987) showed N_2O had no effect on nausea and vomiting in 780 patients undergoing non-abdominal surgery although the patients were older than in previous studies. Kortilla and others (1987) found no difference in 110 patients undergoing abdominal hysterectomy. Hovorka and others (1989) anaesthetised 150 patients for laparoscopy using either isoflurane and N_2O, enflurane and N_2O or isoflurane and air and the incidence of emesis in the first 24 hours in these groups was 54%, 48% and 52% respectively. Overall, the emetic effect of N_2O is still unclear and further study is needed.

It is possible that N_2O is a weak initiator of malignant hyperpyrexia (MH) because this occurred in a patient given only N_2O (Ellis and others 1975). However this must be very rare because N_2O is often used whilst MH is relatively rare. Because N_2O has sympathomimetic effects, it may make the symptoms of MH worse by causing peripheral vasoconstriction which will decrease heat loss through the skin. Its use in known MH susceptible patients is still unclear.

The effects of N_2O on the liver are also uncertain. Minor increases in plasma lactate dehydrogenase isoenzymes concentrations suggestive of mild liver damage were seen in 8 patients receiving 70% N_2O and methohexitone (Prys-

Roberts and others 1983). Two groups were anaesthetised using either halothane or halothane and N_2O in a pressure chamber and plasma hepatic enzyme concentrations increased in the second group (Pratilas and others 1978). This may have been caused by N_2O, but high oxygen concentrations may have prevented it. The postal study of dentists by Cohen and others (1980) showed that the incidence of hepatic disease was increased in both dentists and chairside assistants who had been exposed to N_2O, and that its severity was dose dependent. However, this study has many faults including inaccurate recall, responder bias, and difficulty in obtaining a diagnosis. Lampe and others (1990) found that 60% N_2O when given to 100 patients undergoing hip replacement caused no abnormalities in liver function tests (alanine aminotransferase, bilirubin and alkaline phosphatase). Problems with this trial include small numbers, a relatively short duration of exposure, and the liver function tests may have been too insensitive. More work needs to be done on the effects of N_2O on the liver.

There is no evidence of nephrotoxicity in humans exposed to N_2O although the study of Cohen and others (1980) showed an increase in renal lithiasis in both dentists and their assistants exposed to high concentrations of N_2O which occurred in a dose dependent manner.

There is evidence that N_2O can acutely affect the central nervous system in addition to its chronic actions. It is a cerebral circulatory and metabolic stimulant

(Sabake 1978) with effects additive to those of volatile anaesthetics (Editorial 1987b). This stimulant effect may be dangerous in compromised patients.

In conclusion, N_2O has many potential side effects although several are still unproven and require further investigation. It is expensive to manufacture, store and use. It has caused deaths from both contamination and hypoxia. It can support combustion and may increase environmental pollution of the atmosphere. It is a weak anaesthetic. It has detrimental effects on the cardiovascular and respiratory systems and may interfere with post-operative recovery. It is readily soluble in blood resulting in both diffusion hypoxia and diffusion into body spaces and tracheal cuffs. It may increase the incidence of nausea and vomiting and perhaps influence the progress of MH. It has been implicated in causing hepatic and renal disease and may have adverse effects on the central nervous system.

Why is it used if it has all these side effects? It has several advantages, some of which have already been discussed. It is the only available potent analgesic with a fast onset and recovery. Both induction of anaesthesia and recovery are rapid and the depth of anaesthesia can be quickly changed by adjusting the inspired concentration. It is non irritating and odourless and is pleasant to inhale.

However, its principal advantage comes from the fact that MAC values are additive. Volatile anaesthetics whilst being more potent are also more toxic and N_2O can reduce

the concentrations needed for anaesthesia. Combining inhalational anaesthetics has additive effects on anaesthesia (Cullen 1986). Seventy percent N_2O reduced halothane MAC by 61% (Siadman and Eger 1964) and isoflurane MAC by 60% (Stevens and others 1975). For each 1% N_2O used, the MAC of the added anaesthetic is reduced by approximately 1%. Additionally, N_2O produces amnesia and it is possible that without N_2O there could be an increase in awareness during surgery.

Recently, evidence has shown that N_2O does not have a linear effect in reducing the MACs of other inhalation drugs. Cole and others (1990) showed that in rats, exposure to high concentrations of N_2O had less of an impact in decreasing volatile anaesthetic concentrations needed to maintain a standard anaesthetic depth than would have been expected from its effect at lower concentrations. This suggests that it has more powerful effects at lower concentrations than at higher ones. Possibly there is less advantage in using 70% N_2O rather than 50%.

The main volatile anaesthetics will now be discussed and their advantages and disadvantages over N_2O assessed. New drugs not yet readily available will then be mentioned. Intravenous (IV) techniques, both continuous and bolus methods, will finally be discussed.

Three volatile anaesthetics are used in modern anaesthesia, halothane, enflurane and isoflurane. All are halogenated and possess similar properties although these do vary greatly. All are relatively stable compounds

except halothane which decomposes when exposed to ultraviolet light and certain metals and requires 0.01% thymol as a stabiliser. However, they are all metabolised in the body unlike N_2O. Halothane is metabolised most (20-45%), then enflurane (2.5-8.5%) with isoflurane being relatively stable (0.2%)(Carpenter and others 1986). Metabolism of these drugs produces compounds which are biologically active and these will be described later.

Storage is easier, cheaper and cleaner than N_2O because no cylinders are needed. All are volatile and vaporise in high concentrations at room temperature although separate vaporisers are needed for each volatile drug. Modern vaporisers are accurate although are often only capable of vaporising low concentrations of drug (a safety feature) and this may too low to allow induction or maintainance of a suitable level of anaesthesia without the addition of N_2O. New vaporisers are available which are able to produce a higher concentration of anaesthetic (i.e. 7% enflurane).

Volatile anaesthetics need a carrier gas and normally this is a mixture of N_2O and oxygen. Without N_2O, three mixtures could be used, 100% oxygen or oxygen with nitrogen or air. Pure oxygen has two advantages. No delivery errors can occur and the patient can never receive a hypoxic mixture. Additionally, the patient will always be fully oxygenated and have a large reservoir of oxygen. Its disadvantages include the expense, storage and delivery of oxygen, the increased possibility of fires and oxygen toxicity. It can increase the incidence of

absorption collapse (Winter and Smith 1972) and may injure the lung in a time and dose dependent manner due to production of oxygen radicals (Clark and Lambersten 1971). Whilst most anaesthetics are too brief for damage to occur, using 100% oxygen in prolonged cases may lead to toxicity. Using nitrogen requires extra pipelines or cylinders and it is a hypoxic gas. Oxygen and air mixtures will prevent this problem.

The volatile anaesthetics are not flammable or explosive. All are more potent anaesthetics than N_2O (MACs for halothane, enflurane and isoflurane are 0.75, 1.68 and 1.15) and can be used alone without N_2O to produce an acceptable depth of anaesthesia. However, they all have a lower blood/gas partition coefficient than N_2O (halothane, enflurane and isoflurane have values of 2.4, 1.9 and 1.4 respectively (Stewart and others 1973)) and induction and uptake by these drugs is considerably slower. Two others factors also reduce the speed of induction. None have a 2nd gas effect and both enflurane and isoflurane have unpleasant odours and irritate the upper respiratory tract. All are considerably more soluble in fat than N_2O (Stewart and others 1973) and large body depots can build up resulting in a slow and prolonged recovery.

They have greater effects on the cardiovascular and respiratory systems than N_2O. Halothane and enflurane depress cardiac output whilst isoflurane has only a minor effect (Shimosato and others 1982). All cause some peripheral vasodilation, especially isoflurane. Overall, mean blood pressure decreased 45%, 40% and 25%

respectively with 1.5 MAC when compared to N_2O (Calverley and others 1978). Further increases in inspired concentrations progressively decrease the blood pressure in contrast to N_2O. In compromised patients (shocked, old, etc) this decrease may be fatal. This effect often limits how much of these powerful anaesthetics can be given.

Halothane, and to a lesser extent enflurane, sensitises the heart to catecholamines (both endogenous and exogenous) resulting in an increase in arrhythmias (Munsen and Tucker 1975). Isoflurane may possibly cause a coronary artery "steal" syndrome resulting in myocardial ischaemia. It has been recommended that it should not be used in patients with coronary vessel disease (Editorial 1987a). Cerebral blood flow autoregulation is impaired when more than 0.6 MAC enflurane and halothane is given whilst isoflurane is safe in doses up to 1.1 MAC (Todd and Drummond 1984). The increase in cerebral blood flow that occurs results in dangerous increases in intracranial pressure.

They have similar effects on the respiratory system, depressing ventilation with a decrease in tidal volume and an increase in respiratory rate. One big advantage over N_2O is that they all decrease airway resistance due to a direct bronchodilatory effect (Gold and others 1983). They increase mucus production and depress ciliary movement (Forrest and Chambers 1983). There is a profound dose dependent depression of respiratory control although this is similar to N_2O. There is greater depression of the hypoxic drive with its total abolition when 1.0 MAC of

drug is given.

They have other undesirable side effects. All cause a dose dependent relaxation of uterine muscle resulting in excessive blood loss after anaesthesia for retained placenta, Caesarean operation and evacuation of the uterus (Munsen and Embro 1977). They all cause moderate skeletal muscular relaxation in a dose related fashion due to both a central and a post-junctional effect (Fogdall and Miller 1975). They have no analgesic action and only a general depression of the nervous system will reduce pain.

They also have toxic effects. Enflurane can cause epileptiform changes in the EEG especially with hypocarbia and its use has been contraindicated in epileptics (Soderberg and Grattidge 1977). Halothane is known to cause liver toxicity with an incidence of 1 in 7,000-30,000 halothane anaesthetics (Brown and Gandolfi 1987). This has resulted in the Committee on the Safety of Medicine warning anaesthetists that halothane should not be reused within 3 months in any single patient and that it should not be used if the patient has had a previous history of jaundice or fever after a halothane anaesthetic. Enflurane has also been linked to hepatic damage although the incidence is much lower (Eger and others 1986). As yet, there is little evidence that isoflurane is hepatotoxic. Enflurane is partially metabolised in the body to produce fluoride which can cause renal toxicity and it is suggested that enflurane should not be used in patients with renal failure (Jones 1984). Metabolism of halothane produces bromide ions which

can result in the patient being excessively sedated (Tinker and others 1976). The volatile anaesthetics can initiate MH and using them is contraindicated in patients who may suffer from it (Harrison 1987).

Because all are halogenated hydrocarbons, they can pollute the atmosphere. However, they only contribute about 0.01% of all toxic compounds released into the air (Brown and others 1989). Because their atmospheric lives are relatively short (1-2 years), they are regarded as being "ozone friendly" (Editorial 1989).

In conclusion, these 3 drugs are powerful anaesthetics and have the potential to fully anaesthetise patients without the need for N_2O. They have some advantages over N_2O being stable with long shelf lives, are more convenient to store, can allow greater concentrations of oxygen to be given, cause muscle relaxation, and are bronchodilators. They have no effect on the body spaces and are ozone friendly. However, they also have many disadvantages. They are highly metabolised in the body, require a carrier gas, are expensive, have a high solubility in both blood and lipid and therefore blood concentrations will change only slowly. They are irritant, have no analgesic actions, relax uterine muscles and cause increases in intracranial pressure. However, more importantly, they are powerful cardiorespiratory depressants and can cause organ toxicity and MH. These disadvantages contraindicate their use in certain surgical procedures and also may limit how much can be given in other cases especially when the patient is ill.

New volatile anaesthetic drugs are available (sevoflurane and desflurane) and these may be more suitable. However, it appears that they have little advantage over the present drugs. Sevoflurane has a low blood/gas coefficient (0.6) which gives a smooth induction (Morisaki and others 1988). It is metabolised like enflurane although no organ toxicity has been seen (Cook and others 1975). However, it depresses the cardiorespiratory systems in a dose dependent manner (Kazama and Ikeda 1988).

Desflurane has a low boiling point (23°C) and requires a special vaporiser. It is less potent than the other drugs (MAC 5.4) with a low blood/gas partition coefficient (0.4), is non-irritating and gives a rapid smooth induction with sensitive control of depth (Jones and others 1990). It appears to have cardiorespiratory effects similar to isoflurane (Weiskopf and others 1989).

Could these new drugs fully replace N_2O? Induction and recovery is faster than the older drugs. However, they have similar cardiovascular side effects to the older drugs. Because they have only been used clinically in a relatively few patients, it is possible that they have other toxic effects that have not been discovered. Hence, all the volatile drugs that are either available or soon to be available are not fully suitable to be used alone for all types of anaesthesia.

Will intravenous drugs replace N_2O? At present they are used to maintain anaesthesia in 2 ways, to supplement

inhalational methods or alone as a total intravenous anaesthetic technique (either continuous infusion or bolus injection). Three groups are used: opioids, induction agents and benzodiazepines and other sedatives. Each group will be briefly discussed and then its role in replacing N_2O assessed. An ideal infusion drug should be readily soluble in water, be concentrated (to avoid excess fluid administration), not metabolised by light, not absorbed by plastic and cause no venous damage. It should able to vary anaesthetic depth quickly, have no prolonged action and have little effect on the cardiovascular and respiratory systems (Dundee and Wyant 1988).

Opioids are frequently used before, during and after anaesthesia. They were given as pre-medication in 41% and intra-operatively in 28% of 64,645 anaesthetics (Duthie and Nimmo 1987). Many different types are available which vary in both potency and duration of action. Some are pure agonists and others act as partial antagonists. However, all have adverse effects that limit their use in anaesthesia.

All opioids cause dose dependent respiratory depression and decrease the hypercarbic ventilatory response (Daykin and others 1986). The cough and sigh reflexes are inhibited decreasing secretion removal. Post-operative hypoventilation can occur. Atelectasis is common and carbon dioxide retention can cause cardiovascular problems. Severe hypoxia may result. Respiratory depression is often prevented by naloxone, although this drug has only a short life. Other side

effects of naloxone include nausea and vomiting, restlessness, pruritus, and left ventricular failure. Sequential analgesia (combinations of opioid agonists and partial antagonists) may provide good analgesia with no respiratory depression (Cook and others 1982). Opioids have a sedative effects resulting in prolonged recovery times. Over a 4 year period, 119 cardiac arrests occurred in France which were related to anaesthesia. Half occurred post-operatively and these were mainly due to unrecognised respiratory depression (Pottecher and others 1984).

Chest rigidity can occur when high dose opioids are given which may make ventilation difficult or interfere with surgical access. Induction agents and volatile anaesthetics can reduce rigidity (Christian and others 1983). It is well recognised that the incidence of nausea and vomiting is increased by opioids. For example, women undergoing dilatation and curettage had an increase in the incidence of nausea from 22% to 65% if morphine was given (Rubin and Winston 1950).

Opioids are useful in anaesthesia. They cause no cardiovascular instability and minimally depress the cardiac output and blood pressure. They do not sensitise the heart to catecholamines and have no effect on organ blood flow. They cause no pollution and do not induce MH. They provide good post-operative analgesia and allow artificial ventilation.

Can opioids produce satisfactory anaesthesia when used by themselves? After a lorazepam pre-medication (0.08 or 0.04 $mg.kg^{-1}$), patients were paralysed and an

alfentanil infusion was started. Responses to intubation and painful stimuli were assessed and it was found that lorazepam was needed to reduce the response because alfentanil alone was unable to suppress haemodynamic responses (Hug and others 1988). This is expected because opioids have specific effects at receptors whilst anaesthetic drugs have a more generalised effect. Opioids have too selective an action for general anaesthesia. It appears that other drugs are required with opioids for complete anaesthesia (Editorial 1983).

They do reduce the amount of other drugs required for anaesthesia. If morphine or fentanyl were given, the dose requirements for volatile anaesthetics decreased by 65% (Murphy and Hug 1982). Whilst they may be given with volatile anaesthetics to deepen anaesthesia, cardiovascular depression still occurs because they can interact with volatile anaesthetics and benzodiazepines (White 1985).

In summary, opioids are poor anaesthetics when used alone and other drugs are needed. They have serious side effects, the most important being respiratory depression which may last for many hours.

Benzodiazepines (BZ) have been used in combination with opioids. They are sedatives and in higher doses, hypnotics and act by depressing the excitability of the limbic system due to an agonist action on specific BZ receptors. This is similar to that of the opioids and contrasts with the non specific action of general anaesthetics (Richards and Mohler 1984). Many different

types exist, all with similar properties, but they do vary in their time of onset and duration of action. They are extremely safe having a very wide therapeutic index.

For intravenous anaesthesia, 3 different drugs are used, diazepam, midazolam and lorazepam. All have similar actions although their time of onset and duration of actions are different due to their different solubilities, protein bindings and metabolism. Diazepam is given as an emulsion (diazemuls), midazolam is water soluble and lorazepam requires an organic solvent. Midazolam has a short duration of action when compared to diazepam whilst lorazepam acts for a long time.

All reduce the MAC of inhalational anaesthetic drugs. Diazepam reduced the MAC of halothane by up to 34%, but further doses had no effect suggesting a ceiling effect (Perisho and others 1971). Similar reductions were seen with midazolam (Melvin and others 1982). They have important amnesic properties with midazolam and diazepam giving an intense, short lived amnesia whilst that for lorazepam lasted longer. No retrograde amnesia is seen. (Dundee and Wilson 1980). They have no analgesic properties.

They have no major effect on the cardiovascular system and cause only a mild decrease in systemic vascular resistance (Al-Khudhairi and others 1982) with little effect on cardiac output. Midazolam and diazepam have been safely used in high risk cardiac cases (Kuwar and others 1983). The overall effect is much less than that of the IV induction agents. They have little effect on either

coronary or cerebral blood flow (Hilfibier and others 1980). They have little effect on the respiratory system, but in high doses, will decrease the hypercapnic response (Forster and others 1980). They cause an increase in the incidence of respiratory obstruction when the patient is sedated (Dixon and Thornton 1973).

Therefore they are hypnotic drugs with few side effects. Can they be used clinically in general anaesthesia in place of N_2O? Like opioids, it is impossible to use them as the sole anaesthetic drug because they have no analgesic potential and they have a ceiling on their anaesthetic properties (Pelisho 1971). They can be used as intravenous induction drugs producing a smooth induction although the onset is slow and unpredictable (Halliday and others 1985). They have little cardiovascular instability when used as induction drugs and therefore may be the drug of choice for sick patients. They may have prolonged effects after surgery resulting in delayed recovery. Fentanyl was given with either midazolam or thiopentone for short operations and recovery was prolonged in the midazolam group (Fragen and Caldwell 1983). Specific reversal of the sedative actions of BZ is now possible using flumazanil although, like naloxone, it has a short duration of action and is expensive.

They are commonly used for pre-medication producing anxiolysis, sedation and amnesia. They have been used with opioids in cardiac anaesthesia although awareness is a recognised complication (Hilgenberg 1981). Overall, BZ

have few serious side effects although are poor anaesthetic drugs. They are not used for anaesthesia by themselves, but have been used with other drugs.

The 3rd group of drugs are the IV induction agents. Only a few are suitable for infusion techniques as defined by Dundee and Wyant (1988) although all have been used for shorter cases by bolus injection. Thiopentone rapidly accumulates and repeated doses will prolong recovery. Methohexitone has a faster rate of metabolism although it is still extensively redistributed and can accumulate delaying recovery (Hudson and others 1983). Etomidate was a popular infusion drug until it was found to cause adreno-cortical suppression (Sears and others 1983) and its product licence for infusion has been withdrawn. Althesin was believed to be the best drug available until it too was withdrawn due to hypersensitivity reactions.

Two drugs are used for IV infusions in modern anaesthesia, propofol and ketamine. Propofol is a phenol derivative formulated as an emulsion. It has many features which make it a suitable drug for IV anaesthesia including fast recovery after its use. It causes cardiovascular depression and this can limit its use in compromised patients. Propofol infusions at twice the minimum infusion rate decreased blood pressure more than methohexitone or N_2O (Prys-Roberts and others 1982). It has no known toxic effects and it is recommended for general anaesthesia both with opioids and inhalational agents (Healy 1985).

Ketamine is both a powerful analgesic and hypnotic

allowing it to be used by itself as an anaesthetic. Side effects include emergence delirium and post-operative dreams. Benzodiazepine pre-medications overcome these central nervous effects although delayed recovery may occur. (Lilburn and others 1978).

Total intravenous anaesthesia (TIVA) was initially popular because it was thought that chronic exposure of theatre personnel to inhalational agents (both N_2O and volatile anaesthetics) could have toxic effects. Other reasons for its development include certain operations where it is difficult to administer inhalation agents (access, high oxygen concentrations required, the use of high frequency ventilation, etc) or where inhalational anaesthetics are not safe (cranial operations, etc). The problems of TIVA include awareness, prolonged recovery, cardiorespiratory depression, technical difficulty and cost of new equipment. TIVA is being used more and more and is no longer confined to enthusiasts.

Awareness may be a problem with intravenous anaesthesia especially when modern muscle relaxants are given. Both N_2O and the volatile drugs have good amnesic qualities. Sixty six percent N_2O is more effective than 50% and the addition of enflurane abolished any awareness. (Farnsworth 1978). N_2O may abolish the incidence of awareness (Editorial 1982). Opioids cause no amnesia (Hilgenberg 1981). Grell and others (1970) showed that the addition of 50% N_2O to oxygen-fentanyl prevented post-operative recall in 500 patients. However, in contrast to this, 20% of all awareness cases reported to the defence

unions were due to an abnormally low inspired concentration of N_2O being given (Hargrove 1987) and 70% of these were a result of faulty anaesthetic technique with most involving a N_2O:oxygen:opioid anaesthetic. Is too much reliance being placed on N_2O? Induction agents provide a greater degree of protection. Fifty five patients undergoing major gynaecological surgery received either N_2O (66%) with fentanyl 0.25 mg at induction or an etomidate infusion with air. Temazepam was used for premedication, thiopentone for induction and vecuronium provided muscle relaxation. Incremental doses of 0.1 mg fentanyl were given if clinically indicated. Using the isolated forearm technique, 44% who received N_2O were found to be awake during some part of the operation compared to 7% for the etomidate group (Russel 1986). It appears that N_2O has potent amnesic qualities although awareness can be prevented by other drugs except opioids.

Patients undergoing surgery for major trauma may be especially susceptible to awareness because the patient is often haemodynamically unstable and anaesthesia must be light. Fifty one trauma patients were studied, some received only a muscle relaxant and others an inhalational agent and occasionally N_2O. Ten individuals were aware, 6 having had received no anaesthetic for at least 20 minutes (Bogetz and Katz 1984). Early use of benzodiazepines may have helped to prevent this. Lack of N_2O may therefore result in an increase in the incidence of awareness.

Recovery is theoretically faster when N_2O is used as compared to intravenous drugs. However, N_2O is usually

given with other drugs and recovery is mainly influenced by these drugs. Recovery may be prolonged with longer operations if N_2O is not used, although a fast recovery may not be clinically useful. Recovery is important in short cases. Ninety-four day case patients received either intravenous fentanyl and propofol infusion or fentanyl with enflurane and 66% N_2O. Both groups had similar recovery times, but there was a lower incidence of vomiting without N_2O (Price and others 1988). Therefore, it is probable that awareness and recovery may not be an important problem when N_2O is not used.

There is, as yet, insufficient evidence about the balance between the benefits and dangers of not using N_2O to make an objective assessment about banning its use in anaesthesia. Therefore any opinion must be subjective. There are learning difficulties with any new technique. Anaesthetists are familiar with inhalational anaesthesia and feel able to measure anaesthetic depth accurately when using it. However, a fear of learning new techniques is no reason for failure to adopt superior treatments.

There are certain anaesthetics were N_2O has been proven to be contraindicated. There are indications that it may be toxic to certain individuals including women in early pregnancy and the sick patient. A safe delivery method is important to prevent hypoxia. Its lack of potency requires the use of additional drugs. Theatre pollution can be a problem especially during short cases and because there are suitable intravenous drugs for this type of surgery, a strong case for the abolition of N_2O

may be made in this type of surgery.

With intermediate length cases or with cardiovascularly unstable patients N_2O may still have an important role. A major advantage of N_2O is the number of cases in which it has been given with no obvious detrimental effect. This has been taken as an indication of its safety although the occasional severe problem may have been missed. Its other advantages include it being a analgesic and its cardiorespiratory stability. Recently work by Eger and others (1990) has indicated that some of its potential side effects are not as severe as was once thought. Indeed, he now suggests that it is a safe drug and should still be used by anaesthetists.

More work is needed to investigate both its side effects and its toxic effects. For the latter, how much exposure is required before clinically important consequences occur? Are there identifiable groups of patients who are more susceptible to its effects? Does N_2O contribute to the morbidity of patients by reducing an individual's ability to fight infections and repair wounds? Are sick patients more vulnerable? Does it cause a pollution hazard and are pregnant women especially vulnerable? Are there better methods of preventing MS inhibition?

There are questions still to be answered about N_2O and anaesthesia. Does it increase emesis, can other methods of preventing awareness be used, does it have hepato-renal toxic effects, does it cause increased lung problems and has it detrimental effects on the bowel?

These and similar questions still need to be answered. This thesis has shown that N_2O can have an effect on an important metabolic pathway in lower exposure doses than was previously thought and that this effect varies between individuals. Anaesthetists appear to be unaffected. This work, combined with other studies has made me believe that while N_2O still has a place in anaesthesia, this may not be true for much longer, especially with the development of cheap easily controlled infusion techniques. Instead of N_2O being used routinely for all anaesthetics perhaps it used be reserved for specific indications. Definite evidence of early toxicity or the discovery of a new, safer inhalational or intravenous drug would indeed place its use in anaesthesia in jeopardy.

CHAPTER NINE

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ACKNOWLEDGEMENTS

I would like to thank the Association of Anaesthetists of Great Britain and Ireland for funding both myself and the project, Professor AA Spence for his help and encouragement, Drs Rae, Chambers and Gray for advice and assistance and, finally the nurses, both in theatre and in the wards of the Princess Margaret Rose, City, Western and Eastern Hospitals.

ABBREVIATIONS

AICAR	5 amino 4 imidazole carboxamine transformylase
AMP	Adenosine monophosphate
Atm	Atmospheres
ATP	Adenosine triphosphate
BZ	Benzodiazepines
CO2	Carbon dioxide
CSF	Cerebrospinal fluid
DHF	Dihydrofolic acid
DNA	Deoxyribonucleic acid
dSAM	Decarboxylated S-adenosyl methionine
dTMP	Deoxythymidine monophosphate
dTTP	Deoxythymidine triphosphate
dUMP	Deoxyuridine monophosphate
dU test	Deoxyuridine suppression test
ED50	Effective dose for 50%
FIGlu	Formiminoglutamic acid
FormylTHF	Formyl tetrahydrofolate
FPS	Folylpolyglutamate synthetase
FTS	FormylTHF synthetase
GAR	glycinamide ribonucleotide transformylase
GSH	Glutathionine
GSSG	Oxidised glutathionine
IP	Intraperitoneal
IV	Intravenous
MAC	Minimum alveolar concentration
MethenylTHF	Methenyl tetrahydrofolate
MethylTHF	Methyl tetrahydrofolate
MethyleneTHF	Methylene tetrahydrofolate
MMCoA mutase	MethylmalonylCoA mutase
MS	Methionine synthase
MTA	Methyl thioadenosine
MTR	Methyl thioribulose
N2O	Notrous oxide
PMN	Polymorphonuclear
PPM	Parts per million
RNA	Ribonucleic acid
RR	relative risk
SACD	Sub acute combined degeneration of the cord
SAH	S-adenosyl homocysteine
SAM	S-adenosyl methionine
SCE	Sister chromatid exchanges
THF	Tetrahydrofolate
TS	Thymidine synthase